GENETICS AND CANCER

A collection of papers presented at the thirteenth annual symposium on fundamental cancer research.

LONDON

PETER OWEN LIMITED

PETER OWEN LIMITED
50 Old Brompton Road London SW7

First published in the British Commonwealth 1961

Bound in Great Britain
Printed in the USA

Acknowledgments

The staff of the University of Texas M. D. Anderson Hospital and Tumor Institute gratefully acknowledges the assistance of the American Cancer Society, Texas Division; the Bertner Foundation; the Heuermann Foundation; the State of Texas Department of Health; and the National Cancer Institute, National Institutes of Health, United States Public Health Service, for providing funds for support and publication of this symposium.

Gratitude is expressed also to the members of the 1959 symposium committtee for their efforts in arranging the program. Members of the committee were Felix L. Haas, chairman, Joe E. Boyd, Jr., Leon L Dmochowski, Charles O Doudney, Gilbert H. Fletcher, A. Clark Griffin, Arthur Kirschbaum,* Warren K. Sinclair, and H. Grant Taylor.

This symposium volume was edited and arranged for publication by the following members of the publications department: Russell W. Cumley, Marilyn Abbott, and Joan McCay.

Finally, the staff acknowledges the University of Texas Press and the Printing Division of the University of Texas for their efforts in the production of the book.

* Died May 23, 1958

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Introduction

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Cancer research does not now have, nor is it likely to have in the foreseeable future, the well-defined boundaries of research that one finds in most other fields of medicine or biology. New discoveries and the development of new techniques in many areas of research constantly demand the revision and re-evaluation of existing programs and thinking. It is universally recognized today that cancer research poses problems which require for their solution information from all the various disciplines of biological and medical research. Furthermore, only through the cooperative efforts of groups of basic scientists and medical scientists, and of research groups and clinical groups whose members are highly specialized in a number of different areas, can we expect to answer the questions of neoplastic growth processes. This all-inclusive approach has proved valuable in the comparatively brief time it has been in operation, and during the past twenty years more has been learned about cancer than in the previous two hundred years. However, this type of approach has created the tremendous problem of keeping abreast with the discoveries and information which may be of possible pertinence to the cancer problem evolving from all these areas. It seemed to us here at The University of Texas M. D. Anderson Hospital and Tumor Institute that one way we could be of service to those scientists and practitioners engaged with

cancer would be to hold an annual meeting which would take up one of the facets of the problem and discuss the current research and findings which have bearing on that facet. These annual symposia have resulted from this idea, and we have found the past symposia to be extremely valuable.

This year the thirteenth of these symposia is on the subject of *Genetics and Cancer*. That the subject is most timely is apparent from the fact that two of the most coveted awards in medicine went to geneticists in 1958. The Nobel prize in medicine was awarded to Drs. Lederberg, Beadle, and Tatum for their work on theoretical genetics and the genetics of microorganisms. The Lasker award went to Dr. A. D. Hershey for his work on the genetics of bacteriophages and viruses. The subject of this symposium is a broad one, for it encompasses not only the etiological theories of cancer, but its progression and inheritance factors as well. The program has been designed so that we will discuss recent advances in basic genetics theory and its application to cancer, the experimental genetics of cancerous tissues, and the genetic aspects of human cancer.

Ever since de Vries, in 1901, first advanced the mutation theory, as a result of his studies on the evening primrose, the idea that cancer is caused by gene mutations in somatic cells has received considerable attention. Boveri, in his paper "The Origin of Malignant Tumors," (1914) stated that "such tumors arise as a result of abnormal developments in the chromosomes or chromatin-complex which may be caused in many different ways." As a result of Muller's demonstration that x-rays produce genetic mutations, Bauer in 1928 incorporated the idea of gene mutation into the somatic mutation theory of cancer. More recently, Professor Darlington has incorporated the plasmagene theory into the somatic mutation theory.

Recent developments in animal virus research, and in the seemingly remote areas of bacterial genetics and bacteria-bacteriophage relationships, indicate that the virus theory and the somatic mutation theory of the origin of cancer do not exclude one another. Furthermore, these developments suggest that perhaps we are on the brink of discovering that which oncologists have long sought—a common etiology for many neoplastic diseases. The rapidly increasing evidence indicating that neoplastic changes are accompanied by extensive alterations in the cellular DNA and DNA-containing structures certainly does more than pose the possibility that genetic structures are involved in cancerous changes.

It is also of significance that the great majority of the mutating

agents prove to be quite active carcinogens, and that the majority of them are known to exert their effects by means of their actions on the DNA, or on intracellular materials soon to be incorporated into the DNA. Much of this recent work on the mechanism of mutation induction has been done here at M. D. Anderson Hospital by Drs. Haas and Doudney. They have found that ultraviolet-induced mutagenesis in bacteria is brought about by chemical alteration of the pyrimidine precursors of DNA by ultraviolet rays prior to DNA synthesis. In their experiments, pre-existing DNA was not affected by the ultraviolet radiation so as to cause mutations Their results have been closely paralleled on the carcinogenic side by experiments performed by Dr. Stanfield Rogers at the University of Tennessee. He has found that ultraviolet- and urethane-induced carcinogenesis of certain types in rats is initiated by changes brought about in the building blocks of DNA. These results would seem to indicate that large groups of carcinogens may reduce to mutagens, and that the action of such carcinogens may prove to be fairly direct on the cellular genetic mechanism or its precursors If this is the case and tumor induction is due to a resulting gene modification or elimination of the genetic control of intracellular syntheses essential for normal cell division and function, then it may be possible to develop a rationale for chemotherapy which would allow the control, elimination, or reversal of the cancer process. For as Dr Muller has pointed out, the attitude that just because a certain ailment is proved to be inherited or due to genetic mutation we must regard it fatalistically and set it down as something impossible to influence by environmental means is quite fallacious As direct proof of this we have only to observe the great number of diabetics who are leading perfectly normal lives today

A wealth of experimental data has made it apparent that hereditary factors and the genetic composition of the potential tumorous host play an important part in the development and progression of tumors As early as 1907 Ernest Tyzzer showed that the progeny of mice with mammary or lung cancer were more likely to have these diseases than were the progeny of nontumorous animals. Today, in resistant animal strains, while cancer can usually be produced with the proper carcinogenic agent, it never occurs unless the agent is applied In closely related susceptible strains almost every animal will die of cancer without the application of any apparent carcinogenic agent, and the type of the cancer and the time of its onset can be predicted with great accuracy. Similar factors are known to operate in

the case of some human malignant tumors, such as certain types of intestinal cancer.

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GENETIC THEORY AND CANCER ETIOLOGY

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Plasmagene Theory and Cancer Genesis

C. D Darlington, M.A., PH.D., D SC., F.R.S.
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The Common Denominator

In attempting to understand the cause of cancer there is one question we have to ask first. It is, as we all agree, this: In all kinds of cancer, in all kinds of breakdown of synthetic and mitotic control, is there a common denominator? If there is no common denominator, there is no point in our meeting to discuss all kinds of cancer. If there is a common denominator, all other questions follow from our success in defining it.

Various answers have been given to this question Chromosomes, viruses, and plasmagenes have all been invoked And they all help us to focus our attention on the right points and to express our ideas in the right words.

For example, the idea that abnormal mitosis, giving rise to abnormal chromosome complements, could be responsible for the origin of tumors was foreshadowed by Heidenhain in 1894, but first clearly expressed in 1902 by Boveri This idea implied three hypotheses vital for the approach to the cancer problem. The first was the hypothesis that cancer arises not from continuous but from discontinuous or particulate change It was inevitable that with the development of genetics and biochemistry our views should lean away from fluids, influences, and essences and toward specific particles and their mutation And, in this respect, we have followed Boveri The second hypothesis was that in this particulate change we must distinguish between two genetic components of the cell, the nucleus and the cyto-

effective in differentiation Sewall Wright, in turn, was unaware that I had, two years earlier still, used this term to cover genetic particles in the cytoplasm which passed through the egg in heredity. I was, however, concerned in 1944 in showing that the distinction between agents of heredity, of development, and of infection often breaks down Experiments in breeding peas which revealed the laws of heredity to Mendel had indeed, in my opinion, already begun to reveal their breakdown to Bateson. Sewall Wright's broadening of the use of the term plasmagene, therefore, coincided with my own broadening of the idea.

The two statements will make the story clearer:

> Plasmatic inheritance, like Mendelian inheritance, is particulate. . . . The particles in the nucleus are genes; those in the plastids and cytoplasm may perhaps be treated more rigorously if we also think of them as genes—plastogenes and plasmagenes . . . The possible nature of the plasmagene is indicated by what we know of virus diseases . . . This is to suppose that a virus is not a primitive enemy of nature but just a protein out of place. (Darlington, 1939, pp 121–122)

> It may be, however, that the more or less complete early isolation of the germ line of higher organisms has come about in evolution to maintain *a line of cells* with plasmagenes lacking in prosthetic groups and hence in *specialized* activity but capable in somatic cells of combining with such groups *emanating* from the nucleus to form molecules that multiply thereafter as plasmagenes of a more specialized sort. (Sewall Wright, 1941, p 503)

Both these statements imply that the plasmagene is a genetic particle having a continuity or ancestry going back to the origins of life. Today, however, we are uncertain how far the RNA or cytoplasmic or plasmagene system is dependent on, and how far it is independent of, the DNA or nuclear system. We are uncertain, partly because, as I shall try to show, the origin of cancer represents a shift in the relations of two systems.

The Sequence of Events

First allow me to summarize my own view of the relations of cancer and plasmagenes. (Darlington, 1948, 1949a and b, 1953; cf. Darlington and Mather, 1949.)

The cells of an organism consist of nuclei usually of constant character with respect to their chromosomes—their nucleoli, of course, are not constant—and cytoplasm, which changes in character during

plasm. This was a new idea, and here again we follow Boveri. No one will now, I imagine, wish to confuse these continually interacting but independent foundations of cell life and cell division. The third hypothesis was that the nucleus and not the cytoplasm is the prime mover in the cancer mutation, either the single mutation or the sequences of mutations. It is here that we are in conflict, and here the main issue lies today.

It was the issue between nucleus and cytoplasm that fifteen years ago, in common with several other people, I felt could be decided. I felt that we could exclude the nucleus from the problem of cancer causation. I had, and still have, a great respect for the chromosomes, but their properties did not seem to offer what was needed for the origin and later development of cancer. But the cytoplasm did offer what was needed. My view in this respect had been foreshadowed on specific evidence by Otto Warburg as long ago as 1914 and, more recently, by Graffi and by Haddow, as well as, no doubt, by others. Now, however, there was new and diverse evidence of determinants in the cytoplasm. Their chemical character had been revealed as ribonucleoproteins by Brachet, Caspersson, Claude, and Potter (cf. Darlington, 1942). Their genetic character had been shown, by experiments on plants, animals, and protista, to be conditionally diverse It was for these particles that I had proposed the name of plasmagenes.

The plasmagenes offered us (on the evidence then available) that same paradoxical versatility of behavior which had become a more and more puzzling feature of cancer. In their dependence on the nucleus as well as in their autonomy, in their stability as well as in their mutability, in their diffusibility or infectivity as well as in their fixity, the plasmagenes outside cancer paralleled and provided what was needed inside cancer.

Or, as I put it, in breaking through the established boundaries between heredity, development, and infection, the plasmagenes, which were often potential viruses or proviruses, also broke through the boundaries between general biology and cancer research

Before we broach the cancer problem, it is worth our while to note that the idea of the plasmagene was called forth by the requirement of discoveries in widely different fields of inquiry, both biological and biochemical, at almost the same time. In consequence, I was unaware in 1944 that Sewall Wright, in an illuminating discussion three years earlier, had developed the use of the term plasmagene to cover self-propagating cytoplasmic particles derived from the nucleus and

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come naturally infectious. They will be set going on a selected, adaptive, evolutionary course of life as true viruses.

We must now consider the bearing of new experimental evidence on these views. We must look for parallels between the actions and changes of genetic particles in cancer and in ordinary development.

Experimental Parallels: Plasmagene Differences

Apart from infection there are two kinds of change in conditions which upset the equilibrium between the self-propagating or genetic components of the cytoplasm. One provides a parallel with the primary mutation. Chemical treatment can directly alter the structure of these components. Such treatment may take the form of dyes or antibiotics which prevent the division of one component in a protozoan cell, as with the kinetoplast in Trypanosoma, or cause its mutation, as with the chloroplast in Polytoma (Hoare, 1954). Or the effect may be invisible, as when a mutation is induced in a respiratory enzyme system of yeast by acriflavine (Ephrussi, 1952).

The other kind of change provides a parallel with the secondary changes arising from increase in rate of growth and cell division. Here we find that, both with chloroplasts in Euglena (Darlington, 1949b) and kappa particles in Paramecium (Sonneborn, 1947, 1950), a rate of division of the whole cell surpassing that of a special particle may enable the cell to run away from some of its constituent particles. Thus, the character of the whole cell is altered inherently by a change in the rate of its multiplication Such a change cannot affect the nucleus with its synchronized reproduction—it can affect only the cytoplasm with its unsynchronized reproduction.

These secondary changes enable us to understand how the primary changes come about. We need not, indeed we cannot, suppose that plasmagene mutations take effect directly like nuclear gene mutations. A genetic change in a plasmagene will always give rise to a mixed cell with competition between old and new plasmagenes The analogy will be with cells having two types of plastids, arising either by mutation or hybridization Such plastids have rates of propagation which are conditioned by the genotype of each and its relation with the nucleus (Schötz, 1954). The less favored will, therefore, gradually disappear, with a gradual effect on the properties of the whole cell

The action of irradiation in inducing and causing the develop-

development and differentiation, and hence from tissue to tissue. The character of the cytoplasm depends on self-propagating nucleoproteins—plasmagenes—some of which are permanent in the sense that they pass through the egg, or even sperm, and are transmitted in heredity; while others arise during development and differentiation from the activity of the nucleus. The plasmagenes of development will differ from one tissue to another as a result of cumulative interactions between the nucleus and predecessors of the plasmagenes in the cytoplasm.

In the course of propagation of such a system, errors will occur. *Their occurrence will be subject to the character of the nucleus of the* particular individual. It will be subject to the character of the cytoplasm of the particular tissue at each stage of development. It will also be subject to external conditions and treatments. Changes in one plasmagene or in the balance of several may thus appear to be either spontaneous or induced. And such changes may affect the whole cell so as to stimulate protein synthesis and mitosis just as nuclear changes are known to do.

This primary change in rate of growth of the cytoplasm may require injury for it to be realized in a mature tissue. It will then have a secondary effect, the upsetting of the balance between all the self-propagating components of the cell. Both the plasmagenes and the nuclear genes will be deranged by the increased rate of growth or respiration in the cell, for all genetic particles in the cell are limited and specific in their rates of propagation Thus, when two kinds of particles can be distinguished, as in plastids or kappa particles, they can be seen to compete with one another, with results which depend on growth rates. And the nucleus itself is subject to a whole range of disorders which arise from alterations in the timing of mitosis.

The primary change or mutation will, therefore, lead to a sequence of secondary changes, nuclear and cytoplasmic. The tumor will become heterogeneous Secondary cell lines will be established which will compete and be selected with more and more enhanced rates of propagation until malignancy and metastasis arise.

This is the first step in the theory. The second step is the assumption that the effective plasmagenes will sometimes be capable of artificial transmission by inoculation of cell-free extracts, as with the agent of the Rous sarcoma. And rarely, where the particles concerned lie in superficial cells and can find a vector (as in myxomatosis) or lie in externally secreted cells (as in Bittner's milk factor), they will be-

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terminant through the cytoplasm of the egg is at present without evidence However, the highly specific determination of cancer as between different tissues is one of its most striking characteristics It is, therefore, to plasmagenes of development rather than of heredity that we must turn for the source of the cancer mutation

The distinction between plasmagenes and viruses is of immediate importance, for plasmagenes and viruses evolve under contrasted conditions, and, as we know, they evolve rapidly. A plasmagene depends for its survival on the survival of the group of organisms (the *stock* or race) in which it is maintained by heredity. A virus depends *for* its survival on the survival of the group of organisms *to which it* is *transmitted* by *infection*, and that means natural infection. The *contrasting* situations of heredity and infection are therefore the *foundation* of the contrasting properties of plasmagenes and viruses.

It is in the light of this principle that we have to consider the types of particles significant in cancer. We then find that a large sample of these particles—how large, we are only in process of discovering—is infective under artificial conditions But these particles are not infectious in *nature* They could be viruses but they never have been. The classical *example* is the Rous sarcoma. And the *Rous agent*, as Graffi pointed out in 1940, *arose* by a change, a mutation, in the fowl in which it was discovered.

It is to particles of this description that I gave the name *provirus*, and *the* value of the distinction has been *strengthened* by the later discovery *of the parallel* situation in bacteria where a prophage which is transmitted by *heredity* can become a phage which is distributed by *infection*.

It is in these *circumstances* that we have to consider the remarkable new experiments of *Gross*, *Graffi*, Law, and others, dealing with both spontaneous *tumors* and those induced by *agents of* several types

Gross has shown that *leukemia* can be transmitted by inoculation of cell-free extracts *into* newborn animals. Graffi and others have procured *the same* transmission with solid *tumors* Law and Potter (1958) have proved that *the effect* of irradiation of one tissue can be transmitted by *diffusion* to induce a tumor in another unirradiated tissue, the thymus of a *mouse* (This experiment helps us to understand how lethal *irradiation* can induce viable tumors. It is perhaps always by diffusion from *dead cells to live* ones.) All these experiments have the effect of separating the cell into its two parts and showing that one *is the agent* of cancer production and the *other* is not They make *it clear* that the agent of cancer production, whether

ment of tumors and of leukemia is inherently without parallel in experimental breeding. The time needed in producing the result may be almost indefinitely prolonged. And the dose is usually so high that nearly all nuclei dividing must be put out of action by chromosome breakage. The nuclear basis for propagating a changed cytoplasm is therefore destroyed. The action of irradiation is, therefore, without additional experiments, useless as a means of discriminating between hypotheses.

On the evidence of parallel situations, a sequence of several events is, therefore, likely to be concerned in the plasmagene mutation which gives rise to cancer.

Plasmagenes and Viruses

Some writers have viewed with impatience the distinctions between different kinds of genetic particles in the cytoplasm, and particularly my distinctions, which are so difficult to understand, between viruses, proviruses, and plasmagenes. Lederberg (1952) has promoted them all to "plasmids." Huxley (1958) has reduced them all to "paragenes." Virus workers everywhere would like to describe them all as viruses, masked or active, latent or patent.

Any supposed virus theory of cancer has another accidental advantage. Since Hippocrates, medical training has always been directed toward treatment and cure; this cure was all the more important for Hippocrates—and for many of his successors—since he invented the inheritance of acquired characters, as well as the diffusion principle by which that inheritance was to have come about. To be sure, most diseases which are due to heredity are incurable; but this is something the patient never cares to know and the physician never likes to tell. Lately, therefore, infection rather than heredity, viruses rather than genes, have had an inherent appeal in medical research. When, in addition, we find that viruses, unlike genes, are readily separable from their biological environment, the appeal is powerfully reinforced.

We now know, however, that the situations of particles naturally propagated by heredity, development, and infection are different. They differ in respect to the selective and evolutionary processes to which they have been and will be subjected. Even if chemically the particles are indistinguishable, as they may be, we must, therefore, give different names to them.

Since I have recently (1958) summarized the evidence, I will now confine myself to the conclusions. Transmission of any cancer de-

terminant through the cytoplasm of the egg is at present without evidence. However, the highly specific determination of cancer as between different tissues is one of its most striking characteristics. It is, therefore, to plasmagenes of development rather than of heredity that we must turn for the source of the cancer mutation.

The distinction between plasmagenes and viruses is of immediate importance, for plasmagenes and viruses evolve under contrasted conditions; and, as we know, they evolve rapidly. A plasmagene depends for its survival on the survival of the group of organisms (the stock or race) in which it is maintained by heredity. A virus depends for its survival on the survival of the group of organisms to which it is transmitted by infection, and that means natural infection. The contrasting situations of heredity and infection are therefore the foundation of the contrasting properties of plasmagenes and viruses.

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It is to particles of this description that I gave the name provirus, and the value of the distinction has been strengthened by the later discovery of the parallel situation in bacteria where a prophage which is transmitted by heredity can become a phage which is distributed by infection.

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Gross has shown that leukemia can be transmitted by inoculation of cell-free extracts into newborn animals Graffi and others have procured the same transmission with solid tumors. Law and Potter (1958) have proved that the effect of irradiation of one tissue can be transmitted by diffusion to induce a tumor in another unirradiated tissue, the thymus of a mouse. (This experiment helps us to understand how lethal irradiation can induce viable tumors. It is perhaps always by diffusion from dead cells to live ones) All these experiments have the effect of separating the cell into its two parts and showing that one is the agent of cancer production and the other is not They make it clear that the agent of cancer production, whether

spontaneous or induced, is diffusible, like a cytoplasmic virus. It cannot have the essentially nondiffusible character of a nuclear gene.

These experiments bring animal tumors closely in line with the earlier example of the plant tumors induced by *Bacillus tumefaciens*. Here, also, the inducing agent is graft-transmissible. It also is a provirus (de Ropp, 1947, 1948; Gautheret, 1949; Braun, 1952).

In the light of these discoveries the connections between the different types of cancer also become clearer. Not only do we see that nuclear change is excluded, but we see the whole spectrum of cytoplasmic changes revealed. At one extreme we have the unconditional or natural viruses like myxomatosis and Bittner's milk-transmitted and semen-transmitted mammary cancer. Next come agents which can be artificially transmitted without special precautions, like the Rous sarcoma. Thirdly, there comes a group which requires precautions, especially the use of newly born recipients, to overcome immunity reactions. And, lastly, comes a collection of tumors the transmission of which has not yet been accomplished. In all this series, the distinctions are significant in that they are separable from the common denominator.

Two problems now present themselves: Which came first, the virus or the plasmagene? What happens when the cancer mutation reaches an incidence and a localization of 100 per cent; in other words, when it ceases to be unpredictable?

The first of these questions is, for some of us, intrinsically charged with emotion. To the virologist the virus clearly came first. For the geneticist the plasmagene came first. For most students of cancer, however, it may appear as an evolutionary question and is therefore hardly relevant. The evolution, however, in this case, is happening from day to day. The one thing is arising from the other thing in the origin of each tumor. It is not, therefore, irrelevant.

Let us now look for particular evidence.

Experimental Parallels: Diffusible Plasmagenes

What we now have to discover is whether there is any more specific experimental evidence, outside the field of cancer, deciding whether plasmagenes arise from viruses or vice versa. Earlier I have quoted the instances of L'Héritier's CO_2 resistance agent in Drosophila and Sonneborn's kappa particle in Paramecium. Both these are cytoplasmically inherited. Neither of them is naturally infectious

but both can be made artificially infective. Both may therefore be regarded as proviruses. But both might have been true viruses in the past, having become honest plasmagenes on a doctrine of progress.

To exclude this possibility most rigorously we need examples of plasmagenes which play an indispensable part in the genetic system of the species but are nevertheless diffusible. Do such things exist? There are now two instances, or rather two groups of instances: one is in an animal, the other in a plant; one distinguishes stocks within a species, the other is a means of distinguishing species.

Our first instance of a cytoplasmic particle controlling the breeding system is in Drosophila. In *D. willistoni* there is a naturally transmitted "sex-ratio" character. Males are absent in certain lines. This serves as a reproductive economy and also it favors crossbreeding. Malogolowkin and Poulson (1957) have now shown that this character arises from a lethal reaction between one kind of cytoplasm and several kinds of nucleus. Further, the cytoplasmic variation is due to a particle transmitted only by heredity, indeed, only by the egg, in nature Yet artificially it can be transmitted by infection.

(Parenthetically I must note that the authors use a phrase which appears to contradict my description. Such accidents are easy in this subject. They say of their particle "that it is *essentially* infectious *in nature*" [my italics]. They mean, I believe, that it is *not* essentially infectious and that it is *not* infectious in nature. For it is infectious only by their skillful manipulation It is infectious therefore only in potentiality.)

This lethal genetic particle is, we may say, not infectious but infective. It is not a virus—but, being diffusible, it is a provirus And, since it is transmitted by heredity and is adapted and necessary to the genetic system of the species in which it exists, it is a plasmagene of unimpeachable pedigree

It will be interesting to see from further results how far the nuclear variation which allows some males to survive, and no doubt depends on many polygenic differences, arises from a suppressed propagation of the provirus in females and how far it arises from a viability of males in the presence of the provirus Our evidence from parallel circumstances in Paramecium of Sonneborn, and in *Zea mays* of Rhoades suggests that both types of variation will be concerned.

Diffusible plasmagenes no doubt often distinguish not only strains, but also species of animals Immunity reactions, however, restrict their discovery by grafting, and it is in plants that the critical experi-

ments have been carried out. Repeating Russian work, a Swedish investigator, O. L. Hall (1954), has shown that wheat embryos grafted on the nutritive endosperm tissue of rye are permanently changed. When they flower they can be crossed with rye and set 14 per cent of seed instead of the 3 per cent which is set by plants not so grafted.

Evidently something passes from the rye plant and propagates itself in the wheat, something which is later necessary for the development of hybrid embryos. What is it? This something is diffusible and self-propagating, and characteristic of rye but absent from wheat. It is something nonnuclear but adjusted to the difference between the nuclei of the two species. Its absence is indeed a barrier to crossing the two species, and therefore is something adaptively important and of the same evolutionary significance as the coadapted differences between nuclei and plastids of different plant species. It is a plasmagene.

The symbiotic specificities of species of Phaseolus for their nitrifying bacteria obey the same rules as the crossing capacities of Triticum. When one is grafted on another, the stock confers its specificity on the seedling of the scion (Hoffmann, 1927; Darlington, 1944). This analogy is strengthened by the discovery that symbiotic specificities in clover are cytoplasmically inherited (Nutman, 1949). To describe the particles responsible for these effects as viruses would clearly be out of place. They are plasmagenes which happen to be diffusible; not viruses but, if you like, proviruses.

This kind of specific evidence when combined with the general evidence of the origin of plant and insect viruses makes the evolutionary direction, plasmagene to virus, difficult to avoid.

What is the alternative to this view? It is that all plasmagenes which give rise to viruses are merely latent or masked viruses. They are viruses, potential pathogenic agents, waiting to be uncovered, waiting to be caused to mutate to an active form by accident or by treatment, or by a nuclear gene recombination which follows either inbreeding or crossing. All kinds of plants and animals are liable to contain them, and some must be full of them. When they are carried through the egg, the result is indistinguishable from heredity. But to conform with the hypothesis, this must not be called heredity; it has to be a "vertical transmission of potentially pathogenic agents" (Gross, 1958). This view would be vindicated if a higher frequency of leukemia in the progeny followed inoculation of the parents. But of this, I believe, there is no evidence.

This is a terminological conjuring trick: it is not enough to allow a genetic particle to circumvent the evolutionary laws to which heredity subjects it. For consider before you accept this view, how such an extraordinary situation could have begun. Where did all the viruses which so readily disappear come from in the beginning? Or, to put it in another way, which came first, the host or the parasite? Or do you imagine they did not evolve, but were separately created at the beginning?

There can be no doubt that biologists created the virus before they created the plasmagene, but that does not mean that nature worked in that order. The host must have existed and reproduced its cells with the help of RNA proteins before the virus came to do the same Especially since many of the spontaneous viruses, cancer and other, are not known even to go back as far as the egg. Infection, therefore, contrary to common medical belief, depends on even more complex relationships than heredity.

I thus come back to the view expressed by a series of acute observers in examining the supposed "virus" origins of cancer. One of these I may quote "If one postulates a normal virus occurring in normal cells one had better call it something other than a virus" (Boycott, 1933) Indeed, one had better call it a plasmagene.

The Chromosome Variable

The irregularities of chromosome behavior seen in tumors led Boveri to suggest that these were the cause of malignancy Later, Winge found in tumors the variety of cell types, both polyploid and unbalanced, which should arise from these irregularities. He made the valuable suggestion that competition and selection must occur within the population of different cell lines comprising a tumor. When, however, Koller (1947) observed flourishing mitosis in tumor cells with *much less than the* diploid number, a different complexion was put on these observations As Koller pointed out, such cells would be eliminated in ordinary tissues by their own incompetence. Instead of that they showed *synchronized* mitosis, a sign of cytoplasmic control Their occurrence in active growth and division indicated that the tumor cell is not subject to the usual restrictions on growth and mitosis: it is enjoying privileged treatment.

On this view, the cytoplasm had taken over control from the nucleus in the tumor, and unbalanced cells were being produced as a result of too rapid mitosis, *such* mitosis, in fact, could be directly ob-

served not only in tumors but in pernicious anemia (La Cour, 1944). These cells were continuing in rapid mitosis, not on account of the unbalanced nuclei but in spite of them.[1]

Several instances are known in plants where mitosis which has been put out of step by gene action is associated with chromosome breakage (Darlington and Haque, 1955). Conversely, Koller (1953) has found that solid tumors which regularly suffer chromosome breakage escape from breakage when multiplied under ascites or tissue culture conditions. Again, as each new instance is found where irradiation or chemically induced tumors can be propagated by cytoplasmic extracts, it becomes more and more difficult to believe that chromosome breakage is the effective agent.

There remains one significant field of chromosome action in tumors. The achievement of transplantability, that is, loss of strain specificity, has been found to be correlated with polyploidy in tumor cells (Hauschka and Levan, 1953; Sachs and Gallily, 1956). Its relationship with the inherent properties of tumor origin and dedifferentiation is not, however, clear at the moment. Three possibilities ought to be considered: Is the loss of specificity inherent in polyploidy? Is it the result of genic unbalance such as will usually follow polyploidy? Is it the result of a speeding up of mitosis which will lead to polyploidy as a parallel consequence with change in balance of cytoplasmic particles?

At present, there seems no reason to doubt that variations in chromosome numbers occur in tumors, not because they matter more than elsewhere, but because they matter less, and of course are more frequent than elsewhere. Unbalanced cells of the right kinds survive under specific and novel cytoplasmic conditions *following the onset* of rapid mitosis because the cell has become less dependent on nuclear balance than in regular development. That the nucleus is still of some account we know, since we can stop mitosis by breaking too many chromosomes. And special chromosome complements with special chromosome types (such as dicentrics) then serve as markers by which we can recognize cell lineages, much as color marks lineages in men and cattle (Koller, 1952).

[1] Certain cases of "disintegration" of chromosomes in higher polyploid cells (e.g., Koller, 1956) may now perhaps be interpreted in another way. Ishihara and Yosida (1956) have found that increase from 8x to 32x in regenerating rat cells is accompanied by reduction of chromosome size. Thus, halving of the chromosome threads may compensate for polyploidy (cf. Darlington, 1955).

Three Level Inheritance of Cancer

Cancer differs from diseases of direct genetic determination in the variability of its familial incidence. In an outbreeding human population its incidence is sporadic and only rarely do we find a high correlation in time and place between siblings. But when such populations are selected and inbred, as they can be in other animals, we can produce almost any frequency and any time of onset beyond sexual maturity that we want Even without selection the limit of 100 per cent incidence is reached in the uniform first generations of hybrids in animals and plants (cf. Kehr and Smith on Nicotiana, 1954).[2]

These properties are shared by true cancers and leukemias. They may also be expected to be shared by pernicious anemia and by quite unrelated noninfectious distempers, such as eczema, the irregularities of whose inheritance, arising from two superimposed variables—the nucleus varying in the individual and cytoplasm varying in development—become, therefore, of interest for cancer research In all these conditions the inheritance that is being studied, the effective variable that is being selected, is nuclear and genic

In none of these conditions does inheritance enable us, in my opinion, to distinguish between the mutations of genes and those of plasmagenes that are controlled and determined by the nucleus. It is the secondary mutability and the cytoplasmic transmissibility of the tumor that enables us to exclude the nucleus as the vehicle of mutation

Summary

Fifteen years ago the genetic and chemical evidence had shown that there were self-propagating particles in the cell parallel with those in the nucleus but consisting of RNA protein instead of DNA protein These particles, which had been described as plasmagenes, were (in different conditions) the basis, primarily, of development and differentiation, and secondarily, of some heredity and some infection Changes in these particles could be held responsible for the origin of all types of tumor.

Today the new evidence of diffusibility in plasmagenes shown by breeding experiments closely parallels the new evidence of artificial

[2] At this point certainty seems to contradict the uncertainty of ordinary cancer origins, and direct determination (by infection or heredity) appears to replace conditioned mutation But experience shows that it is merely the end point of a series

(but not natural) infectivity in cytoplasmic extracts of a variety of spontaneous and induced tumors. This parallel reinforces the view that plasmagenes can give rise to viruses. It also reinforces the view that tumor agents always arise from plasmagenes whether or not they are transmissible as proviruses. Further, it excludes the possibility that a mutation of nuclear genes is directly concerned. The variety of chromosome types in tumors is indeed evidence that, within the range of their variation, the nucleus is subordinated to the cytoplasm.

All diseases which, like the cancers of man, show an absence of true infection combined with a high indeterminacy of inheritance, are probably subject to the double indeterminacy of nuclear recombination and plasmagene mutation.

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(but not natural) infectivity in cytoplasmic extracts of a variety of spontaneous and induced tumors. This parallel reinforces the view that plasmagenes can give rise to viruses. It also reinforces the view that tumor agents always arise from plasmagenes whether or not they are transmissible as proviruses. Further, it excludes the possibility that a mutation of nuclear genes is directly concerned. The variety of chromosome types in tumors is indeed evidence that, within the range of their variation, the nucleus is subordinated to the cytoplasm.

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The Role of Somatic Mutation in Neoplastic Growth

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Our first problem is a problem of definition: Somatic mutation has been so loosely defined that it can encompass almost any change in cell heredity. Let us examine the degree to which studies on genetically favorable organisms allow a redefinition of the concept and its re-evaluation for our discussion of carcinogenesis.

Before doing this, we must ask whether the nature of the malignant process demands any specific etiology. In other words, does the phenotype of the neoplastic cell itself impose any restrictions on the possibilities for somatic mutation? In answering this question, I should like simply to recall these distinctive characteristics—the continued mitosis of these cells, not influenced, as are normal cells, to differentiate by their relationships with other cells in the organism; the peculiar invasive character which is so important in the metastatic process; and the changes in metabolism which are the biochemical concomitants of these peculiarities.

For any of these particular changes in phenotype, there is no necessary direct relationship to a specific change in cell heredity. It is true that the changes in metabolism might be accomplished by a decrease in the number of mitochondria per cell, if one were to suppose that these were autonomously replicating structures and mutated to less effective types. However, all that we know of replication under such circumstances—that is to say, in such cases of apparently independent intracellular components as the kappa particles in Paramecium or the

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tissues of interest, which can be observed as mosaics, with patches of tissue clearly differing in their constitution from the surrounding cells. For example, the eye color of Drosophila and the endosperm color in maize are useful in this regard and have formed the basis of some of the more elaborate studies of mutation. Using such characters, the early workers on induced mutations made comparisons between the frequencies of induced mutations in somatic tissues and the germ line. The net conclusion from these early studies was that, by and large, the frequencies of the mutations induced per chromosome were of the same order of magnitude in the germinal and in the somatic tissue. The implication was, therefore, that the process of mutation in both types of tissue might be comparable.

Without reviewing the earlier works in any detail (see Muller, 1954), I should like to note that these conclusions are perhaps premature. They were restricted in the number of loci studied, and, moreover, it now appears at least possible that with such mutagens as the alkylating agents we may find differences in the sensitivity of different tissues and chromosome regions (Fahmy and Fahmy, 1957)

The characteristics of somatic mutation are best studied in the cases of frequently mutating loci; a variety of these have been studied in different organisms Here, the general methodology has involved, as has been mentioned, the use of stable mutants discernible in a tissue. The use of linked markers affecting different characteristics in the same type of cell can make feasible the study of the nature of the chromosomal change which causes the type of mutation observed. Following its use originally in the studies of chromosomal elimination in such mosaic types as gynandromorphs, this kind of analysis led to the discovery of such phenomena as somatic crossing over.

Somatic Mutation in Maize

The first detailed studies of somatic mutations were carried out in plants, which have the great advantage that the somatic tissue can give rise to germ cells, and the proof of the mutational character could be obtained by conventional genetic techniques. The classic case is variegated pericarp in maize, originally studied by R. A. Emerson (1917). It happens at the present time to be at the center of the most recent advances in our information about mutation phenomena. Taking up the study of variegation at this locus, Brink and his associates (Brink, 1958a) have shown that it falls into the category of the cases analyzed by McClintock in studies which led to her develop-

plastids in plants—tells us that their replication can be affected in a variety of ways. Even for the mitochondria, therefore, any particular etiology is not required to give the change in metabolic phenotype characteristic of the tumor cell. Similarly, the stimulus to mitosis can be encompassed as the result of a viral etiology, in which one would suppose that the competition for the precursors of the mitotic structures was so changed in the cell, as a result of the multiplication of the virus, that these structures were facilitated in their replication. If one considers conventional gene mutations, from what we already know about the interrelationships in the metabolic nexus, a variety of types of mutation might be conceived which would lead to this same result of continued mitosis. Furthermore, the special phenotype of invasiveness itself is distinctive in the different types of malignancy; and one would expect that a whole variety of cellular changes might be involved in this also.

All this is not surprising to the geneticist. He is familiar with the fact that mutants with a very similar appearance result from changes at quite different loci; and roughly similar end effects may be caused by disturbances at any of the steps along a biosynthetic pathway. This being the case, the fact of the malignant cell as a different entity from the normal cell constrains us to no specific type of etiology.

Methodology for the Study of Somatic Mutation

The primary difficulty in the study of mutation in somatic cells is that the methods usable in the germ cells for the study of random mutations are not available for somatic cells except (quite recently) in tissue culture. In somatic cells, the mutants ordinarily detectable are limited by the characteristics of the cell type; the detection of mutants on a random basis, made possible by such techniques as the study of lethals, is not easily feasible here. In microorganisms the study of biochemical mutants changed from the normal in nutritional requirements has provided, as is well known, a technique comparable to the study of lethals. The recent studies of differences in nutritional requirements of cell lines from different tissues indicate differences in the cellular heredity of these types and may give us a spectrum of mutations which will be comparable to those obtained in the germ cells. This, however, is still in the future

But in the cell populations of a developing organism the technique has not yet been worked out which will accomplish this result. What we must do, instead of the random tests, is to make use of mutants already available affecting visible characteristics in the different

nomenon. Especially, the time of occurrence in ontogeny is dependent upon the number of these "controlling" loci present in the nucleus. There is an integration of the genetic behavior: What is sufficient to preserve the stability of the locus in one cell generation is no longer adequate at a later stage given the same Activator-Dissociator complex. More than this, Brink (1958a, 1958b) working with another of the color loci in maize—the R locus—has been able to show a so-called transallelic effect which he has called paramutation. Here the mutability of the opposite allele in a heterozygote is determined by its partner, at least in one specific case The frequent somatic rearrangements necessitated by the maize analyses, moreover, need not be random. All in all, the phenomena are sufficiently striking to make one consider such possibilities in other cell types

So far, the integrations of which I have been talking depend on nuclear factors These—whether one calls them Activators, Modulators, Dissociators, elements—are essentially a class of genetic units whose function is the control of chromosome behavior. Nothing in the existing information requires the assumption of autonomous cytoplasmic factors for these phenomena.

Somatic Mutation in Drosophila

Somatic mutation in animals is more difficult to study. Here we have no meristematic tissue which would form mutant sectors to be tested in the germ line by conventional Mendelian techniques What can be explored is a parallel of somatic behavior with that in the germ line. This type of analysis was carried out by Demerec (1941) in *Drosophila virilis* many years ago Working with a background in the mutable genes of plants, he was able to demonstrate in a series of mutable genes in *D. virilis* that the mutation process was extremely rich and complex. In the case of the mutable "miniature" wing locus, in addition to a variety of stable alleles which controlled the size of the wing, there were alleles mutable either in the somatic or in the germ line or in both He was further able to show that changes between one "state" of the gene and another could happen—changes between a mutability in the soma to mutability in both germ line and soma, and so on The striking additional discovery was that modifiers of the mutability of this locus existed elsewhere in the chromosomes. Thus, the mutability in the genome was conditioned by the interaction of a whole system of factors. This was something discovered during the thirties, quite a long time ago; and, as is evident, it contains the same principles that we have seen more recently exemplified in

ment of the concepts of controlling elements. If one examines closely, as McClintock did, an ear of maize from certain special strains, the individual grains of corn are mosaic for certain color characters. She was able to show that the different types could be interpreted as resulting from the combination of two separable mechanisms. One is a local change, for example, the inhibition of pigment formation specifically associated with a particular locus; and the other a pattern involving changes at a sequence of loci along the chromosome, due to successive chromosome breakages and reunions in a series of mitotic cycles. The element responsible for a local mutability, she found to be separable from the unit responsible for the pigment formation. She termed these controlling elements "Dissociator loci" in general. When the Dissociator locus was adjacent to the color factor, its activity was inhibited, and no pigment formed. The variegation in these strains, *the spotted kernels, she showed to be associated with the removal* of the Dissociator locus from its proximity to the color factor, to a place elsewhere in the genome (McClintock, 1956).

For the Dissociator locus to be effective, another element, according to McClintock, the "Activator locus," must be present in the genome. As an extreme effect, the region of the Dissociator locus formed unions between sister chromatids at mitotic divisions leading to bridges at the succeeding anaphases. Thus, a cycle of breakage and fusion is initiated; cells in such a line of descent have duplications or the complementary deficiencies for special regions of the chromosome involved, and show variegation for the visible effects of the genes located in them.

Changes in the pattern of the variegation, she showed, were due to changes in the number of these Activator loci present in a given nucleus as well as to changes in the state of the locus (or its position). *The effect of the Dissociator adjacent to a color-producing or marker* gene is an example here, and a number of different cases have been studied.

The evidence for this complex series of phenomena was obtained using orthodox Mendelian techniques. In the cases when the Activator disappeared from its locus next to the marker gene, she was able to show that it was present elsewhere in the same nucleus and to pick up its presence by appropriate tests, using the Dissociator to mark the presence of the Activator. Brink has been able to show that similar elements, which he has called "Modulators," are present in the variegated pericarp.

Somatic mutation in maize is thus a most sensitive and labile phe-

nomenon. Especially, the time of occurrence in ontogeny is dependent upon the number of these "controlling" loci present in the nucleus. There is an integration of the genetic behavior: What is sufficient to preserve the stability of the locus in one cell generation is no longer adequate at a later stage given the same Activator-Dissociator complex. More than this, Brink (1958a, 1958b) working with another of the color loci in maize—the R locus—has been able to show a so-called transallelic effect which he has called paramutation. Here the mutability of the opposite allele in a heterozygote is determined by its partner, at least in one specific case. The frequent somatic rearrangements necessitated by the maize analyses, moreover, need not be random. All in all, the phenomena are sufficiently striking to make one consider such possibilities in other cell types

So far, the integrations of which I have been talking depend on nuclear factors. These—whether one calls them Activators, Modulators, Dissociators, elements—are essentially a class of genetic units whose function is the control of chromosome behavior. Nothing in the existing information requires the assumption of autonomous cytoplasmic factors for these phenomena.

Somatic Mutation in Drosophila

Somatic mutation in animals is more difficult to study. Here we have no meristematic tissue which would form mutant sectors to be tested in the germ line by conventional Mendelian techniques. What can be explored is a parallel of somatic behavior with that in the germ line. This type of analysis was carried out by Demerec (1941) in *Drosophila virilis* many years ago. Working with a background in the mutable genes of plants, he was able to demonstrate in a series of mutable genes in *D. virilis* that the mutation process was extremely rich and complex. In the case of the mutable "miniature" wing locus, in addition to a variety of stable alleles which controlled the size of the wing, there were alleles mutable either in the somatic or in the germ line or in both He was further able to show that changes between one "state" of the gene and another could happen—changes between a mutability in the soma to mutability in both germ line and soma, and so on The striking additional discovery was that modifiers of the mutability of this locus existed elsewhere in the chromosomes. Thus, the mutability in the genome was conditioned by the interaction of a whole system of factors. This was something discovered during the thirties, quite a long time ago; and, as is evident, it contains the same principles that we have seen more recently exemplified in

the studies of the maize mutants. However, in the *D. virilis* analysis, there was no evidence of chromosome aberration; the salivary gland chromosomes were normal, and no linkage disturbance was evident. There is no sign of the frequent transposition of elements which is so striking in the maize mutation picture; whether this part of the phenomenon is peculiar to maize, or whether it is an intrinsic feature of this kind of mutation process and not easily demonstrable in the *D. virilis* studies, cannot be decided.

There is another type of somatic change in Drosophila which does involve chromosome rearrangement, analyzed most extensively in *Drosophila melanogaster*. This was first discovered by Muller (1930) on the analysis of mosaic mutants in the progeny of x-rayed flies and has turned out to be uniformly associated with a special type of chromosome rearrangement (Schultz, 1936; review in Lewis, 1950). The result of this specificity has been to show certain regularities which have proved useful in the attempt to find some general principles. The point of major importance is the involvement of special regions of the chromosomes, the heterochromatic regions, which participate in these rearrangements. It turns out that a locus abnormally placed next to a heterochromatic region will become variable in its expression. In the few cases where the heterochromatic regions contain marker genes, these too show a variable expression.

Let us look at a few examples that display the characteristics of these processes, worked out by a large number of investigators over quite a few years. Consider first the manifestation of the activity of the white locus. This controls the pigmentation of the eyes, of the testis sheath, and of the Malpighian tubules of the fly. In the white-eyed mutant, the extreme of the series, pigment is absent in all these places. Other alleles run the gamut of colors from a tinged yellow to a red. This is the classic case of multiple allelism in Drosophila. In recent years the analyses of the characteristics of this locus have shown that, like all those intensely investigated, the white locus is complex and contains a minimum of four to five elements (Lewis, 1952; Judd, 1958).

In the rearrangements in which the white locus is juxtaposed to the heterochromatic regions, the result depends upon a variety of factors. Essential is the distance of the white locus from the heterochromatic region to which it is transposed. The existence of a variety of rearrangements in which the white locus is at different distances from the different heterochromatic regions of the chromosomes has allowed

a rather intensive analysis (see Lewis, 1950, for references). The pattern of the variegation serves as a guide here. This varies greatly in the different rearrangements. In some, large patches of tissue are mutant, others remaining the original wild type. In others, patterns of a fine pepper-and-salt arrangement appear, with mutant spots on a red (normal) background or with lighter patches on a darker mutant background. The Drosophila eye develops from a rudiment, composed initially of a few cells, which then multiply and subsequently form the numerous facets which make up the eye. It is evident that differences of pattern must be considered with respect to the timing of the mutational process in development. In a recent study, Becker (1957) has made an analysis of the cell lineage of the eye by inducing somatic crossing over, which we will examine presently. From his analysis he concluded that the upper and lower halves of the eye originally derive from two separate cells; they have, in other words, a different cell lineage Interpretations of the patterns observed in the variegation in this way are useful: In those cases where large patches occur (early in development) there seems to be an orientation of the original cell division Usually the wild type tissue is found in the lower half of the eye, as if the changed daughter cell were more likely to be oriented toward the prospective upper half.

One additional item is the localization close to the white locus of other genes which also affect characteristics of the eye, such as the arrangement of the facets. By using these markers, the identification of the sequence in which the changes have occurred with respect to the heterochromatic block becomes possible. Thus, we have not only the white locus itself to act as an indicator of the variegational change in which the different elements give a sequence of sorts, but also the "roughest" locus immediately to the right on the chromosome map. In a rearrangement in which the heterochromatic regions are placed to the left of white and therefore the *roughest* is more distant, the large patches of mutant tissue in the eye, in which change of expression of the white locus has occurred, are smooth Only in the small patches (thus, those that occurred at later stages in the development of the eye) is the roughest locus changed. When, on the contrary, the break which adjoins the heterochromatic region is to the right of roughest, and the roughest locus, therefore, is closer to heterochromatin, the larger patches are chiefly roughest, and within these roughest patches different gradations of the white locus are found. We have to deal, therefore, with a sequence of changes in which each

step determines the nature of the behavior at the next cell generation.

Furthermore, the change at the white locus depends not only on the distance but on the type of rearrangement. It may go directly to white, as in certain cases; in others, the initial change may be to one of the darker alleles. The lighter patches in the eye are descendants of cells from these dark areas. In general, it appears that where the patches are large—in other words, where a change has occurred early —the most extreme departure from the wild type is to be found.

This heterochromatin-induced variegation is extremely sensitive to modifiers. The most powerful modifying factors in this system, as might be expected, are changes in the relative amounts of heterochromatic regions. The addition of an extra Y chromosome (chiefly heterochromatic) will make a profound change in the expression of the variegation due to such a heterochromatin rearrangement. The extent of the change depends, of course, on the type of rearrangement. This may be illustrated best by considering again one of the variegated white types. In this case, in the original rearrangement, the so-called white-mottled 4, a single Y chromosome in the male shows the characteristic variegated phenotype. With the addition of an extra Y chromosome, the eye is practically red with a few brown specks. With a derived rearrangement, which gives practically white eyes in the presence of a single Y, the result of adding an extra Y is rather different. Instead of the practically normal red, the eye color is now still mottled and rather dark. Only when two extra Y's are added in this case does the suppression of the variegated phenotype occur. There are also changed Y chromosome types, such that a single Y has the effect of two normal ones.

These effects of the Y resemble in principle the dosage effects of the Activator locus in the variegated phenotypes of maize. In maize, with increasing doses of Activator, the mutational effect on the Dissociator locus occurs at successively later stages. Since the removal of the Dissociator locus is necessary for the expression of the dominant, the result is more recessive ("mutant") tissue at higher Activator doses. A somewhat similar situation can be found in the Drosophila cases where the locus being observed is originally in the heterochromatic region and now, due to the rearrangement, adjoins the euchromatic portion of the chromosome. When this happens, the addition of the Y chromosome makes for an intensification of the mutant rather than the wild type condition. If one were to make a comparison between the maize and the Drosophila cases, it would

seem as though in maize the unstable pericarp locus behaves as if its normal site were in a region comparable to the heterochromatic regions of Drosophila; but this is an embroidery which we are not at present in a position to take too seriously.

The whole variegation system is one of extraordinary plasticity. The modifiers which can be found are enormously diverse. Some of them are located in heterochromatic regions, others at regions not normally considered as heterochromatic—perhaps in the intercalary heterochromatin that has been discussed on occasion (see Hannah, 1951, for references). However, even within these modifiers, there is a degree of specificity that recalls the specificities in some of the maize mutants. For example, one of the modifiers of the mottled white already discussed, located in the second chromosome, is dominant to a certain degree. It changes the phenotype of the mottling from a rather dark white allele to a lighter grade. However, when the mutant is homozygous, the white-mottled 4 becomes almost completely white. The homozygous individuals are sterile, and in the absence of white-mottled 4 they have a normal eye color. When an extra Y chromosome is added to such individuals, however, they die at an early stage of development. We thus have a locus revealed which forms part of the integrated system already indicated; but here a highly specific one, for the same modifier which changes the variegation of the white-mottled has not been found to affect other types of variegation; which to the contrary the Y chromosome affects most potently.

Aside from the timing of the variegation process, there is another factor which indicates an interplay between the nuclear and cytoplasmic conditions: namely, the fact that in certain cases the modifiers of the variegated phenotype have a maternal effect. In other words, the character of the variegation during the development of the embryo is determined by the cytoplasm of the egg in which development starts. This has provided a most important clue to the analysis of the nature of the process involved.

From Muller's first paper on, the speculations regarding the nature of the variegation process involved considerations of the possible effects *on replication* of the genes involved in the rearrangement. The examination of the giant chromosomes of certain of these arrangements showed changes in structure amounting in some cases to a practical absence of the loci involved. Less extreme cases consisted of an intensification of the affected bands which measured the amount

of ultraviolet-absorbing material, most likely DNA. From this, a process of change of structure to this final disappearance appears to exist. This series of phenomena suggested that *the nucleic acids in the bands were influenced by the metabolism of the heterochromatic regions, and conversely, that these regions were influential in controlling the nucleic acids of the cell* (Schultz, 1956, contains earlier references).

In our laboratory in the past several years we have attempted to provide some direct chemical evidence by studying the nucleic acids of the egg (Levenbook, Travaglini, and Schultz, 1958, I). By comparing eggs from normal females with those from females containing extra heterochromatic regions, we found that the presence of an extra Y chromosome in a female may lead to a change in the constitution of the RNA of her eggs. Moreover, this change is correlated with concomitant changes in the amounts of the various precursors of the nucleic acids present in the egg cytoplasm (Levenbook, Travaglini, and Schultz, 1958, II). What this implies is that the Y chromosome itself carries an influence on the synthesis of the various precursors of the purine and pyrimidine bases, and by so doing can exert an influence on the nature of nucleic acid synthesis.

The direct implication of such a process in the change of phenotype in the variegated cells themselves has come from studies of antimetabolites. The extent of the variegation is changed considerably by the use of such an antimetabolite as amethopterin, and, most important for our present discussion, this influence can be reversed when thymidine is present in the medium. It follows, therefore, that a process involving thymidine synthesis is a sensitive link in the variegation phenomena (Schultz, 1956). We shall return to this in its context *for the discussion of carcinogenesis.*

The important point at present before we go on to our next topic is to note that we have here a greatly malleable system, one presumably concerned with gene replication, and modified by feed-back processes which control integration in the nucleus as a whole. These occur in such widely diverse organisms as maize and *Drosophila* Some of the so-called gene conversions in microorganisms may be similar also. They show a mutational process not by any means random, but *highly directed, and dependent upon the factors of the* rest of the nucleus before any particular type of mutation is displayed. It is obvious that cases of genetically controlled susceptibility to cancer could find an accommodating hypothesis in this type of explanation.

SOMATIC CROSSING OVER

Before we go into this further, however, it is desirable to consider one of the simpler cases of genetic variation due to the change in the nucleus, namely, somatic crossing over Here also we will find that the condition of randomness in an organism does not hold, but that even somatic crossing over is dependent on the type of tissue, the stage of development, and a whole host of concomitant factors In fact, at the very outset, Stern's analysis of somatic crossing over in Drosophila (Stern, 1936) was contingent on the fact that this process is vastly *increased over the normal frequency* in the presence of the so-called Minute factors, one of whose most striking effects is a decrease in the rate of development Even in these cases, the time of development at which the somatic crossing over occurred, as well as the tissue in which it occurred most frequently, were dependent not only on the Minute itself but also on other factors, such as the chromosomal constitution, etc.

Thus, the picture of the nucleus of the cell and its chromosomal apparatus as a fixed structure, with gene mutations or other genetic changes resulting from random bombardment, appears as an artificial construction, which need no longer alienate pathologists from the somatic mutation hypothesis. This random point of view, as I have just said, is not valid even for so definitely chromosomal a circumstance as somatic crossing over. Quite recently, Becker (1957) in Germany has reinvestigated radiation-induced somatic crossing over. Making a more detailed analysis of cell lineage than was previously available, he has concluded that the occurrence of crossing over in somatic cells as a result of x-radiation is not simply a direct one- or two-hit phenomenon, but some more indirect effect on the cell. In a similar vein, Brosseau (1957) has made an analysis of the effects of temperature upon somatic crossing over in Drosophila. Studying particularly the mosaics in the abdominal tissue of Drosophila, he has shown that an effect of temperature on the frequency of somatic crossing may be demonstrated at two different stages. One occurs very early, relative to the development of the organ. The other occurs quite late, at approximately the time at which the cell divisions which give rise to the mosaics are taking place In Brosseau's analysis it appears that there is an early effect, interpretable as influencing a predisposition on the part of the cells at a much later stage of development toward the occurrence of somatic crossing over. The evidence for this is fairly simple If there were an effect on the early stages directly,

the patches of mosaic tissue in the abdomen would be large. However, if the frequency of small patches increases due to an early temperature shock, it is evident that the effect is a facilitation of the process in the descendent cells.

A similar and even more striking case of a delayed effect on the occurrence of a chromosomal abnormality was found by Brown and Hannah (Brown and Hannah, 1952; Hannah, 1955) in their studies of the irradiation of ring X chromosomes in Drosophila. Here they found the frequency to be influenced greatly by the age of the mother at the time of radiating the eggs in which the abnormal divisions later occurred. What this means is that the condition of the egg cytoplasm in the older eggs was of importance for the character of the cleavage divisions.

Implications for Carcinogenesis

Aside from their intrinsic interest, the point of mentioning these cases is the definite lesson that they have for the theory of carcinogenesis. If mutation is a random process, equally likely to occur in all cells, one has to seek *ad hoc* explanations for obvious facts in the natural history of the cancer: Specific organs are affected and they are affected according to their intrinsic nature. Mutation followed by selection is a possibility which the actual histology of the carcinogenic process does not discourage. Although contingencies of selection are rather difficult to evaluate, it is not difficult to consider as extremes cases in which only the cells stimulated to mitosis would form mutants, of which some could assume the malignant phentoype and have a selective advantage; or conversely, cases in which most cells excepting only those stimulated to mitosis would be destroyed—their products serving perhaps as an additional mitotic stimulant.

The most vigorous attempts to test the mutation hypothesis have been made in comparisons of the mutagenic effects of substances with their carcinogenic effects. Smith (1958), for example, has studied tumor formation in Nicotiana hybrids. In certain hybrids of Nicotiana, tumors can be induced by irradiation, and in the same plants the frequency of somatic mutation for color factors can be studied. Smith found as one might have expected that the somatic mutation and tumor formation ran quite different courses.

In a similar way, Burdette found no correlation between the mutagenic effects of substances and their effects on susceptibility to tumor formation in Drosophila. I should perhaps mention that in our own laboratory we have studied the effects of a number of car-

cinogens on both morphogenesis and on the processes of somatic mutation which I have already described (Schultz and Bischoff, 1953). The effects on morphogenesis and in certain cases on the formation of melanotic tumors were rather striking. But we did not find any comparable effects on the heterochromatin-induced variegation. Nevertheless, such notorious mutagens as the various ionizing and ultraviolet irradiations and the nitrogen mustards are, as is well known, definitely carcinogenic in mammals. But how seriously is one to take the failure of some active carcinogens to be profoundly mutagenic in such organisms as Neurospora or Drosophila (Burdette, 1955)? Obviously, the negative result is not too informative; yet by the same token, the correlation between mutagenesis and carcinogenesis may only be a circumstantial association.

It is here that the results we have been discussing have their value. Once it is determined that mutation in somatic cells is not necessarily a random process and the idea of specificity of mutagenic events in diverse cell types becomes not too frightening, the negative results lose significance. The problem becomes one which must be attacked in its own place, in the specific context of the cell in which the tumors originate and, more importantly, in the context of the relation between chromosome behavior and differentiation as controlled by the genetic history of the cell.

Nuclear Differentiation and Mutation

Thus far I have stayed pretty close to the conventional concepts of somatic mutation. However, we must now consider more closely the relation of these to other types of changes in cell heredity. The thought that mutation of the genetic material could be regular enough to account for the differentiation process has occurred to many people and been discarded by most of those to whom it has occurred. There seemed to be no conceivable mutational processes sufficiently regular to satisfy the requirements of differentiation. This, of course, if one examines it closely, is a fallacious argument. The processes of normal development have been stabilized by an evolutionary history which has rejected most of the errors that could occur. One cannot expect directly to equate the possible changes that might occur in the genetic information transmitted from cell to cell during embryonic development, with the types of errors that we now call mutations. Most of these conceivably are part of the evolutionary experimentation that has been rejected in the normal developmental process. The types of possible change in the genetic material which

constitute the problem of differentiation in the nucleus raise questions of semantics when the relation of this differentiation to the mutational process is considered. The fact that each type of cell is specialized, with only the activities of the cellular substances *in vivo* to guide us as to the genetic information that they carry, makes the problem difficult. We cannot judge what transformations occur and what possible degree of reversibility exists. For example, when the immature germ cell is transformed into the sperm, what happens to the genetic information carried by these chromosomes, whose constitution changes so considerably in the process? The only concrete evidence that we have at the moment derives from the experiments on nuclear transplantation carried out by King and Briggs (1956) which show fairly clearly that irreversible changes occur as the tissues specialize during development. Whether these irreversible changes are in the nature of mutational changes, that is, definite changes in the potential information carried by specific segments of chromosome, or whether they are actual losses of material, is something difficult to say. They may be replacements of special codes of information by others which have no meaning for the cell in which they reside—so-called nonsense DNA. Indeed, my own speculation, as regards the nature of the heterochromatin-induced variegation, has been more or less along this line; namely, that adjacency to the heterochromatic regions induced a change in the type of nucleic acid which no longer permitted the specific manifestation of the gene in question It is not difficult to imagine a system of nuclear differentiation in which such changes would be regular and cell specific, with the end result that for each type of cell a requisite population of information carriers was present (Schultz, 1947, 1958).

At least a superficial alternative to this point of view may be found in the recent analyses of the specificity with which changes in structure (Beermann, 1956; Breuer and Pavan, 1955) and, indeed, in disproportionate synthesis of DNA (Rudkin and Corlette, 1957; Stich and Naylor, 1958), *occur specifically in the chromosomes of different* tissues in definite functional circumstances. These analyses so far are restricted to the giant chromosomes of the Diptera. However, one may see in them a useful principle that may have wide application (for example, Schultz, 1959). These are the phenomena of so-called gene activation, and again these are not necessarily reversible. Indeed, they may be irreversible, and so could lead to the type of nuclear differentiation observed in the nuclear transplantation experiments.

The distinction may eventually involve a definition of gene activation as maintaining a constant pattern of DNA, changed in its biosynthetic effects by associated substances. Mutation here would involve a change in the kind of DNA. Now, what do all these considerations have to do with the role of somatic mutation in neoplastic growth? It is fairly evident that if one is to consider somatic mutation as an important element in this process, the somatic mutations must be of a specific type. According to our current predilections, they must be mutations affecting the nucleic acid metabolism of the cell; and if one continues to restrict the hypothesis, they may be mutations in the heterochromatic regions, if these are viewed as regions specifically involved in the control of nucleic acid synthesis.

However, if nuclear differentiation exists, and it seems now that it must, the fact of the matter is that these somatic mutations must occur in cells which may differ in their nuclear constitution. If this is the case, is it necessary to postulate somatic mutations at all? My own view is that a reasonable possibility which does not involve special *somatic* mutations in the primary initiation of carcinogenesis may be found (Schultz, 1947, 1958) One begins by considering the necessity for the regeneration of cell structures, particularly in the cytoplasm, after exposure to the influence of carcinogens. These influences have their chemical expression in the phenomena leading to the so-called protein-deletion hypothesis. They lead to the loss, during the process of carcinogenesis, of the specific cytoplasmic attributes of the cell, concomitant with the stimulation of this cell to a mitotic cycle. Here the nature of nuclear differentiation is of utmost importance: I have suggested that it may be of such a nature that in each cell type only a special complement of loci maintains its specific activities. Stimulated to mitosis, without their normal cytoplasmic environment, the possibility is apparent that in the competition for substrates, the mitotic rather than the functional aspects of cell metabolism will be favored. This, the early stage of carcinogenesis, permits us the opportunity for subsequent violent changes in the chromosomal constitution In such a population, obviously, somatic mutations may take place, and among them selection for the most viable constitution would occur In the nature of things, the phenotype which we regard as the most malignant would be most highly selected. How progression evolves differs, as pathologists emphasize, from tumor type to tumor type. The characteristics of the tumor are determined not only by the selective value of malignancy, but by the cell of origin as well On this basis, one may more than justify the current preoccupation

constitute the problem of differentiation in the nucleus raise questions of semantics when the relation of this differentiation to the mutational process is considered. The fact that each type of cell is specialized, with only the activities of the cellular substances *in vivo* to guide us as to the genetic information that they carry, makes the problem difficult. We cannot judge what transformations occur and what possible degree of reversibility exists. For example, when the immature germ cell is transformed into the sperm, what happens to the genetic information carried by these chromosomes, whose constitution changes so considerably in the process? The only concrete evidence that we have at the moment derives from the experiments on nuclear transplantation carried out by King and Briggs (1956) which show fairly clearly that irreversible changes occur as the tissues specialize during development. Whether these irreversible changes are in the nature of mutational changes, that is, definite changes in the potential information carried by specific segments of chromosome, or whether they are actual losses of material, is something difficult to say. They may be replacements of special codes of information by others which have no meaning for the cell in which they reside—so-called nonsense DNA. Indeed, my own speculation, as regards the nature of the heterochromatin-induced variegation, has been more or less along this line; namely, that adjacency to the heterochromatic regions induced a change in the type of nucleic acid which no longer permitted the specific manifestation of the gene in question. It is not difficult to imagine a system of nuclear differentiation in which such changes would be regular and cell specific, with the end result that for each type of cell a requisite population of information carriers was present (Schultz, 1947, 1958).

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with population genetics on the part of tissue culture workers and cell biologists in cancer research.

Obviously, the degree to which somatic mutation can be influenced by extraneous circumstances, and its specificity, allow a considerable latitude in the application of this hypothesis. Obviously also, one can consider both viruses and autonomous elements in the cytoplasm (if they indeed exist) as mutagens of a sort or as gene activators, or as Tjio and Östergren would like to believe, as activators of the heterochromatic system itself. All these possibilities are, in *effect, details. The fact of the matter is that the change of cell type* in tumor formation involves a change in cell heredity; and the different systems in the heredity of a cell involve the nucleic acids of one type or another. This being the case, one can hardly escape some form of the somatic mutation hypothesis of tumor origin.

ACKNOWLEDGMENTS

Aided by Grants from the American Cancer Society, Inc., New York, N.Y., and by Grant C-1613 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md.

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Lysogeny, Transduction, and Cancer Genesis

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Neoplastic transformation of somatic cells involves a sudden and irreversible change in their hereditary properties which allows them to escape the regulatory mechanisms of the organism. For the last thirty years two apparently opposed theories, the mutation theory and the viral theory, have aimed at interpreting the essential features of malignancy. Whereas the mutation theory ascribes the primary cancerogenic event to an internal change in the genetic apparatus of the cell, in the virus theory the primary determinant of malignancy is an element external to the cell. This conceptual distinction between heredity and infection for a long time appeared as irreducible.

As a consequence of the progress made in the apparently remote field of bacterial genetics, the concepts of heredity and infection no longer appear irreconcilable, but may be considered merely as two different aspects of the same basic fact—the presence in a cell of a specific genetic structure, whether inherited or acquired from an external source.

LYSOGENY

Much of our knowledge in this respect has come from the study of lysogenic bacteria. These bacteria are able to produce infectious bacterial viruses (bacteriophages) in the absence of infection by an

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grated with the genetic apparatus of the host cell and replicates in harmony with it.

When sensitive bacteria are infected with a bacteriophage, the DNA of the phage, i.e., its genetic material, is injected into the host (Hershey and Chase, 1952). In the case of temperate phages, the genetic material of the phage can then undergo two different series of events. It can either enter the vegetative state which leads to lysis of the infected cells and to production of infectious particles (lytic cycle); or it can establish itself as a prophage and give rise to a clone of stable lysogenic bacteria (lysogenization). The relative frequency of each response is under the influence of genetic as well as environmental factors

Conversely, the genetic material of the phage can leave the prophage state and enter the vegetative state. In cultures of lysogenic bacteria this event occurs spontaneously in a small fraction of the population (from 10^{-5} to 10^{-2} per cell generation, depending on the type of phage) and is lethal for the bacteria in which it occurs (Lwoff and Gutmann, 1950). With certain lysogenic strains the transition from the prophage to the vegetative state may be induced at will in the totality of a bacterial culture by exposure to chemical or physical agents such as ultraviolet light (Lwoff, Siminovitch, and Kjeldgaard, 1950) Those agents which have proved effective in such induction of lysogenic bacteria are agents which also are known to be mutagenic or carcinogenic in other organisms (see Lwoff, 1953; Jacob, 1954). Once a prophage has entered the vegetative state, the series of events which follow are identical to those which occur in the lytic cycle upon infection of sensitive bacteria with the same bacteriophage (Jacob and Wollman, 1953).

The cycle of a temperate inducible bacteriophage is represented in Figure 1.

The Genetics of Lysogeny

In lysogenic bacteria, the genetic material of the phage (the prophage) is "integrated" with the genetic apparatus of the host cell in such a way that both behave as a *single unit* of reproduction. As to the exact nature of this integration, it could only be investigated by genetic methods. This became possible when it was found by E. Lederberg (1951) that the K12 strain of *Escherichia coli*, in which genetic recombination had been demonstrated (Lederberg and Tatum, 1946), was lysogenic for an inducible phage, λ, and that non-lysogenic mutants of this strain could be isolated. Although the early

external virus. They therefore possess and transmit to their progeny, from generation to generation, the hereditary power to produce and release an infectious agent. The bacteriophages so released, or temperate bacteriophages, are able to multiply in and to lyse bacteria of sensitive indicator strains. They also are able, after infection of such bacteria, to give rise to new lysogenic systems. Lysogeny is therefore a clear example of a heritable property which can be acquired by infection.

These properties of lysogenic bacteria, though recognized very soon after the discovery of lysogeny, more than thirty years ago, were not readily understood, and the same controversy which has existed in cancer research between the proponents of the mutation theory and those of the viral theory also took place among students of bacteriophage. The existence of lysogenic bacteria, however, posed the problem of a possible relationship between heredity and infection, and as early as 1928, when comparing the properties of bacteriophages with certain cases of transfer of hereditary characters in bacteria and the transmission of malignant tumors, Eugène Wollman could write: "The two notions of heredity and of infection, which appeared to be so perfectly distinct and in some way incompatible, happen . . . in certain conditions, to be almost confounded."

It was not until 1950, with the work of Lwoff and his collaborators, that the nature of lysogeny and its genetic significance became fully appreciated. The essential features of lysogeny are by now too well known to require a detailed description. They will therefore be only briefly summarized. Additional information may be found in several reviews (Lwoff, 1953; Jacob, 1954; Bertani, 1958; Jacob and Wollman, 1959).

The Properties of Temperate Bacteriophages

Lysogenic bacteria perpetuate hereditarily the genetic information necessary to produce a specific type of bacteriophage. This information is carried by a noninfectious structure, which Lwoff and Gutmann (1950) have called *prophage*. A temperate bacteriophage may thus exist in three different states.

In the infectious state, the bacteriophage is an organized structure whose genetic material (DNA) is enclosed in a protein coat. This coat endows the particle with stability and infectivity.

In the vegetative state, the genetic material of the phage multiplies actively and directs the synthesis of protein phage constituents.

In the prophage state, the genetic material of the phage is inte-

analysis of crosses involving lysogenic and nonlysogenic bacteria pointed to a chromosomal determinism of lysogeny (Lederberg and Lederberg, 1953; Wollman, 1953; Appleyard, 1954), the results were not unambiguous. Only after a better understanding of the mechanisms of bacterial conjugation and an improvement of the methods of mapping bacterial genetic loci had been attained (Hayes, 1953; Wollman, Jacob, and Hayes, 1956; Jacob and Wollman, 1958a), could the chromosomal location of the prophages be firmly established (Wollman and Jacob, 1954; Jacob and Wollman, 1957).

Each type of prophage occupies a specific position on the bacterial chromosome. It therefore behaves as a genetic unit of the lysogenic bacterium. Between the genetic material of a temperate phage and the chromosome of the bacterium there thus exists a specific correspondence or homology. A prophage, however, is not an intrinsic part of the bacterial chromosome. It is neither substituted for an homologous part of the chromosome of the nonlysogenic bacterium, nor is it inserted into the continuity of the chromosome. It is an extrinsic genetic structure, which is added to the genome of the bacterium and fixed in an unknown but specific way to a specific site of the bacterial chromosome. It can be gained or lost as a whole, and, under certain circumstances (action of inducing agents, conjugation, etc.) it can leave its chromosomal site and start replicating in an autonomous fashion (Jacob and Wollman, 1958b). Since the genetic material of a phage is DNA, in the prophage state as well as in the infectious or vegetative states (Stent, Fuerst, and Jacob, 1957), any model of the physical structure of the bacterial chromosome will have to provide for means of assuring the specific binding of the prophage DNA to the DNA of the bacterial chromosome.

The Prophage as a Cellular Genetic Unit

The presence of a prophage on its chromosomal site endows lysogenic bacteria with certain phenotypic properties which distinguish them from homologous nonlysogenic bacteria. One of these properties is the ability to produce a particular type of bacteriophage, but this is a potential property of the bacterial population, since its expression in any single bacterium is lethal (Lwoff, 1953). A property which is expressed in each lysogenic bacterium is immunity, i.e., the specific resistance conferred by a prophage against infection by homologous or closely related bacteriophages. Other phenotypic expressions of the presence of a prophage include properties which, at first sight, would appear to bear no relation to bacteriophage infec-

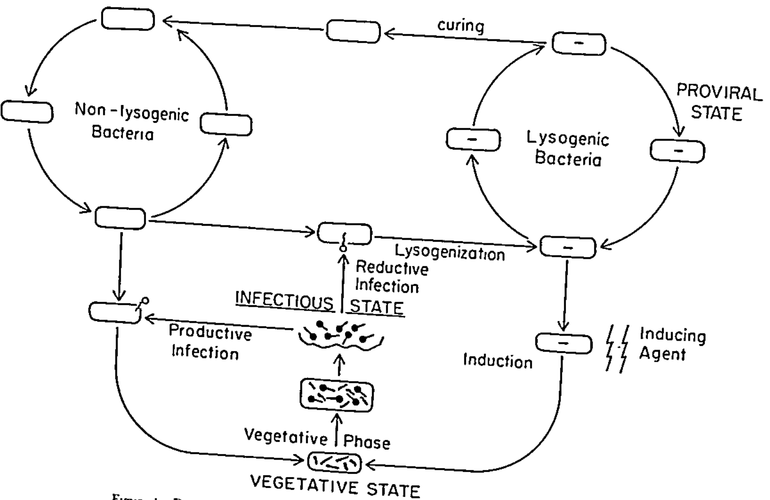


Figure 1 Diagrammatic representation of the cycle of a temperate bacteriophage.

been derived experimentally from normal lysogenic bacteria, it would be extremely difficult to assess that certain of their genetic properties are under the control of a prophage.

Temperate bacteriophages thus possess the properties of both typical viruses, in their extracellular infectious state, and of bacterial hereditary determinants, in their integrated prophage state. Mutations may occur which suppress any one of these aspects. Virulent phages cannot establish themselves as prophages and are typical cytopathogenic viruses; while the defective prophages, which cannot develop into infectious particles and sometimes have even lost the ability to enter the vegetative state, can hardly be distinguished from normal bacterial structures.

Transduction

Bacterial viruses, besides being able to confer certain hereditary properties to the bacteria in which they establish themselves as prophages, also are able, in certain cases, to act as vectors of genetic material from one type of bacteria to another. This phenomenon of genetic transduction, discovered by Zinder and Lederberg (1952), consists in the inclusion, into bacteriophage particles, of small genetic fragments of donor bacteria, which fragments are thereafter transferred, upon infection, to recipient bacteria. In contrast to lysogenic conversion, the characters thus transmitted are those of the donor bacteria, the phages themselves acting merely as carriers of genetic segments from one strain to another (Zinder, 1953).

Generalized, Nonspecific Transduction

In this type of transduction, the first to be discovered, certain strains of phages are able to transmit any of the genetic characters of the donor bacteria to bacteria of the recipient strain. Only a few closely linked bacterial loci can be transferred together in a single phage particle, and the probability of transmission of any given character per phage is small (about 10^{-5}). It is worth mentioning that among 14 temperate phages whose chromosomal location has been investigated, the only one which could not be localized on the K12 bacterial chromosome is a bacteriophage which is able to perform this type of transduction (Jacob and Wollman, 1958b).

Limited, Specific Transduction

This type of transduction was discovered by Morse, Lederberg, and Lederberg (1956a, b) with inducible phage λ, whose prophage

tion. The best known cases of such lysogenic conversions are the formation of diphtheria toxin by lysogenic *Corynebacterium diphtheriae* (Freeman, 1951) and the production of new antigens by certain lysogenic strains of Salmonella (Iseki and Sakai, 1953). If it were not for the fact that these specific transformations can be accomplished at will by lysogenization with the corresponding phage, such hereditary traits could hardly have been ascribed to the presence of a prophage.

As any other genetic structure, the genetic material of a phage exerts two main functions: It ensures its own replication and it directs the synthesis of specific compounds (bacterial enzymes, phage constituents, etc.). It is of the greatest significance that the way in which these two functions are achieved varies according to the state of the phage genome, that is, *according to its relationships with the bacterial genome.*

In the vegetative state, the genetic material of the phage replicates autonomously at a pace which differs from one phage to the other, but which is always faster than the pace of replication of the bacterial genome. It directs the synthesis of the diverse phage components and also of bacterial products such as toxin in diphtheria bacilli (Barksdale, 1958) and antigens in Salmonella (Uetake, Luria, and Burrous, 1958); but it either fails to elicit the synthesis of the cytoplasmic mediator of immunity or it directs the production of a suppressor of this hypothetical substance.

In the prophage state, the genetic material of the phage replicates in harmony with the bacterial genome. No phage material is formed (Miller and Goebel, 1954), but the substance responsible for immunity is synthesized (Jacob and Wollman, 1956a).

Prophage mutations occur, which block some of the steps involved in the development of a prophage into infectious particles (Jacob and Wollman, 1956b; Jacob, Fuerst, and Wollman, 1957). Such defective lysogenic bacteria, which have lost the ability to produce phage, still retain the properties conferred on them by the presence of the prophage. In inducible defective systems, the action of an inducing agent, such as ultraviolet light, still brings about the death of the bacteria, but no infective particles are released. Superinfection with normal phages, however, allows the release of a mixture of normal and defective particles. In defective lysogenic bacteria the genetic material of the phage can thus be perpetuated only in the prophage state. The phage has lost its infectiousness and behaves therefore as a genetic determinant of the host. Had such strains not

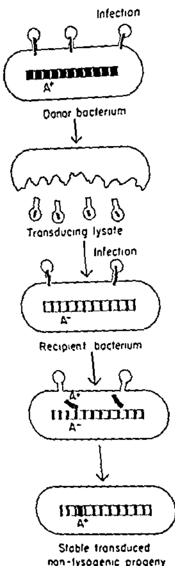


Figure 2 Diagrammatic representation of transduction. A—Generalized non-specific transduction, B—Limited specific transduction.

is located on the chromosome of *E. coli* K12, in the vicinity of a series of loci which control the ability to ferment galactose. After induction of lysogenic Gal+ bacteria (but not after infection), a small proportion of the bacteriophages released (about 10^{-6}) are able to transduce the Gal+ character, and only this character, to Gal- recipient bacteria. This low frequency of transduction (Lft) leads to the formation of clones of unstable Gal+ bacteria from which Gal+ and Gal- progeny segregate. When such lysogenic heterogenotes, which carry both the Gal- and the Gal+ alleles, are in turn induced, they liberate bacteriophages which are able to transduce the Gal+ character at high frequency (Hft). The association which exists between the λ prophage and the locus to which it is linked on the bacterial chromosome therefore persists, in this case, during the whole cycle of multiplication of the phage (Weiglé, 1957).

About half of an Hft lysate is composed of normal λ particles which are unable to transduce, and half of defective λ particles which transduce the Gal+ character. In the defective transducing λ phages (λ dg) a segment which amounts to about one-fifth of the phage genome appears to have been replaced by a segment of the bacterial chromosome (Arber, Kellenberger, and Weiglé, 1957). Transduction by λ dg therefore exemplifies a case where a genetic bacterial segment has become an integral part of the genome of a phage and replicates as a part of this genome in the prophage state as well as in the vegetative state. When a λ dg particle lysogenizes a Gal- bacterium, the Gal+ segment remains part of the prophage and is not incorporated into the recipient chromosome. The transduced heterogenote segregates stable Gal-, nonlysogenic bacteria by loss of the defective prophage. Occasionally, recombination may occur between the Gal+ exogenote and the bacterial chromosome, giving rise to stable nonlysogenic Gal+ recombinants.

The two known modes of transduction which are summarized in Figure 2 appear therefore to be very different The generalized type resembles transformation as studied in Pneumococcus or Hemophilus (see Hotchkiss, 1955), where any genetic character of a donor strain can be transmitted to a recipient strain by means of free DNA extracted from the donor. The restricted type, on the contrary, is a special case of lysogenization and thus resembles lysogenic conversion. However, recent results (Luria, Fraser, Adams, and Burrous, 1958) would indicate that both types of transduction could be mediated by defective phage particles. They would thus not differ in the mechanism by which genetic material can be transferred from one bac-

The Sex Factor of Bacteria

Conjugation in E. *coli* takes place between bacteria of opposite mating types. Certain strains (F^- or females) appear to be devoid of the sex factor F. They never mutate to the F^+ (or male) state, but can acquire the F^+ character upon conjugation with F^+ bacteria (Lederberg, Cavalli, and Lederberg, 1952). Upon its transfer to F^- bacteria, the sex factor appears to replicate autonomously and at a faster rate than the bacterial genome. $F^+ \times F^-$ crosses exhibit a low frequency of recombination (Lfr) but F^+ bacteria can mutate to different types of Hfr (high frequency of recombination) types in which the sex factor is integrated and can be localized at *the distal end of* the Hfr chromosome. Moreover, the presence of the sex factor in the integrated state appears to prevent the replication of the same factor in the autonomous state. The sex factor of bacteria has thus the properties of an episome which can be either absent or present and when present can be either autonomous or *integrated* (Wollman and Jacob, 1958).

The Colicinogenic Factors

Colicins are antibiotics of protein nature which are produced by enteric bacteria and are active on strains of *E. coli* (Fredericq, 1957, 1958). Bacteria which have the hereditary property of producing colicins are called colicinogenic bacteria. Colicinogeny is a stable character. Mutations from noncolicinogeny to colicinogeny have never been observed. Noncolicinogenic bacteria can acquire the colicinogenic character only by contact with colicinogenic bacteria, and this acquisition appears to take place through conjugation. Genetic analysis of bacterial crosses has shown that in male Hfr strains the colicinogenic factor is located on the bacterial chromosome, but that after its transfer to female bacteria it is able to replicate autonomously (Alföldi, Jacob, Wollman, and Mazé, 1958). A colicinogenic factor can thus be either absent or *present*, and when present, it can be either autonomous or integrated.

In their mode of action on sensitive bacteria, colicins resemble bacteriophages in many respects (Fredericq, 1953). Colicins can be best compared to the tail proteins of virulent bacteriophages, whereas colicinogenic factors would bear some resemblance to the genetic material of temperate bacteriophages (Jacob, Siminovitch, and Wollman, 1953). If the parallel between colicins and bacteriophages is of any significance, one could imagine that these two antibacterial

terium to another, but by the events which occur in the donor bacterium in the one case, and in the recipient bacterium in the other. Whether few or many bacterial characters may be transferred by a certain phage would depend upon whether or not the corresponding prophage occupies a specific position on the bacterial chromosome (Jacob and Wollman, 1958b). The type of response of the recipient bacterium (stable transduction or formation of heterogenotes) would depend upon the relative abilities of the transducing phage to establish itself as a prophage or to allow recombination to take place between its exogenote segment and the bacterial chromosome (Luria, Fraser, Adams, and Burrous, 1958).

The Episomes

Situations found in the study of lysogeny thus exemplify cases where the genetic material of a virus can hardly be distinguished from the normal genetic determinants of the host, whereas situations found in the study of transduction demonstrate that a segment of genetic material of the host can become an integral part of the genome of a virus. In both cases, however, the genome of the virus conserves its identity: It can be acquired or lost as a whole, it can replicate in an autonomous fashion, and, even when integrated, it is fixed on the chromosome and not incorporated into it.

The genetic material of a phage, in the present state of evolution of both bacteria and bacteriophages, is a genetic structure which must be considered as distinct from the genetic material of a bacterium. The genetic material of the phage is an extrinsic structure for which there is no allelic segment on the bacterial chromosome, and thus differs from the normal genetic units of the bacterial genome. It may be either absent or present, and when present it may either be in an autonomous state (vegetative state) in which it replicates at its own pace, or it may be in an integrated state (proviral state) in which it is replicated in synchrony with the bacterial genome. Moreover, the presence of the phage genome in the integrated state prevents the autonomous replication of a nonintegrated phage genome. The term of *episomic elements* or *episomes* has been proposed for genetic elements which share similar properties (Jacob and Wollman, 1958c).

Studies in bacterial genetics have shown that three classes of genetic elements, which would appear at first sight to be completely unrelated, have the properties of episomic elements. They are the temperate bacteriophages, the sex factors of bacteria, and the determinants of colicin production.

The Sex Factor of Bacteria

Conjugation in *E. coli* takes place between bacteria of opposite mating types. Certain strains (F^- or females) appear to be devoid of the sex factor F. They never mutate to the F^+ (or male) state, but can acquire the F^+ character upon conjugation with F^+ bacteria (Lederberg, Cavalli, and Lederberg, 1952). Upon its transfer to F^- bacteria, the sex factor appears to replicate autonomously and at a faster rate than the bacterial genome. $F^+ \times F^-$ crosses exhibit a low frequency of recombination (Lfr) but F^+ bacteria can mutate to different types of Hfr (high frequency of recombination) types in which the sex factor is integrated and can be localized at the distal end of the Hfr chromosome. Moreover, the presence of the sex factor in the integrated state appears to prevent the replication of the same factor in the autonomous state. The sex factor of bacteria has thus the properties of an episome which can be either absent or present and when present can be either autonomous or integrated (Wollman and Jacob, 1958).

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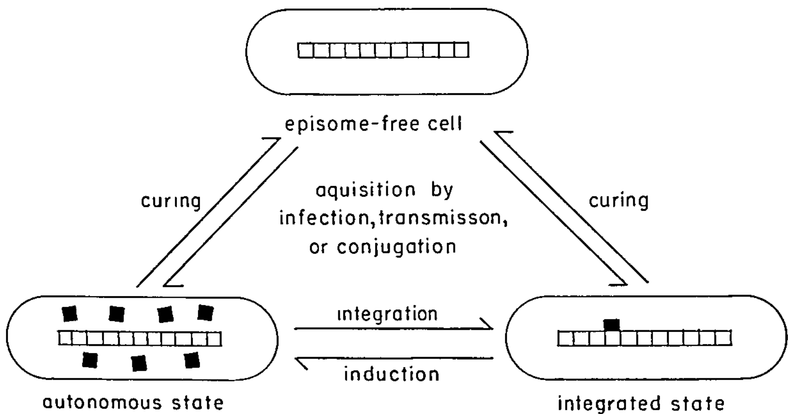


Figure 3 Diagrammatic representation of episomic behavior.

agents are phylogenetically related. A bacteriophage could be visualized as an episome which, in the autonomous state, is able to enclose itself into the products the synthesis of which it directs, whereas a colicinogenic factor is devoid of this property. Whether one should consider the colicinogenic factors as viral genomes which, like defective phage genomes, have lost the ability to undergo maturation, or as a landmark in the evolution of bacteriophages is a question of personal taste. A diagrammatic representation of the behavior of episomes is given in Figure 3.

The genetic analysis of bacterial crosses thus leads to the recognition of a class of genetic elements, the episomes, which are intermediary between the intrinsic genetic determinants of the cell chromosomes, the extranuclear genetic structures or plasmids (Lederberg, 1952), and the extrinsic genetic structures such as viruses. Bacterial episomes comprise very diverse elements which range from typical viruses, as the bacteriophages are, to apparently normal structures, such as the sex factor of bacteria Whether such genetic elements may exist in higher organisms is unknown, although the "controlling elements" described in maize (McClintock, 1956) appear to have similar properties.

Conclusion

The developments of microbial genetics have increased considerably our knowledge of the basic mechanisms of heredity. They also lead to a re-evaluation of our concepts and of our vocabulary as exemplified by the extreme difficulty which sometimes exists in distinguishing infection and heredity. The bearing of this conceptual re-evaluation on the hypotheses which can be formulated as to the genesis of cancer is too obvious to require emphasis.

Although lysogenic bacteria are the only known example of integration of the genetic material of a virus into the genetic apparatus of the host at the chromosomal level, there are indications that other viruses are also intracellularly integrated. Such cases are represented by the hereditary "viruses" of Drosophila which are responsible for the sensitivity of these flies to carbon dioxide (L'Héritier, 1958) and for the "sex-ratio" character (Malogolowkin and Poulson, 1957). In the case of virus-induced tumors, and in particular in that of the Rous sarcoma (Rubin and Temin, 1959), there is also evidence that the virus is transmitted intracellularly in the course of cell division and in some way integrated into the genetic apparatus of the infected cells. For such viruses which are intracellularly perpetuated from cell

generation to cell generation the term of *moderate* viruses (in analogy with temperate bacteriophages) has been adequately proposed by Dulbecco (1958), because the exact relationship of such integrated viruses with the genetic apparatus of the host cells is still unknown. It is indeed to be expected that the mechanism of viral integration may be different in various systems, particularly according to whether the genetic material of the virus is DNA or RNA.

The number of cases where viruses have been shown to play a role in the establishment of malignancy increases, as the means of detection of such viruses improve. The fact that the number of such cases is still limited is perhaps not sufficient to eliminate the possibility that genetic elements having some properties in common with viruses might be involved in the genesis of neoplastic growth. Until the hopeful prospects of achieving the genetics of somatic cells prove substantiated, the examples brought to light in the study of bacterial genetics, and in particular the behavior of episomic elements, might serve as useful models in the analysis of cancer genesis.

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generation to cell generation the term of *moderate* viruses (in analogy with temperate bacteriophages) has been adequately proposed by Dulbecco (1958), because the exact relationship of such integrated viruses with the genetic apparatus of the host cells is still unknown. It is indeed to be expected that the mechanism of viral integration may be different in various systems, particularly according to whether the genetic material of the virus is DNA or RNA.

The number of cases where viruses have been shown to play a role in the establishment of malignancy increases, as the means of detection of such viruses improve. The fact that the number of such cases is still limited is perhaps not sufficient to eliminate the possibility that genetic elements having some properties in common with viruses might be involved in the genesis of neoplastic growth. Until the hopeful prospects of achieving the genetics of somatic cells prove substantiated, the examples brought to light in the study of bacterial genetics, and in particular the behavior of episomic elements, might serve as useful models in the analysis of cancer genesis.

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FUNDAMENTAL ASPECTS OF GENETICS IN CARCINOGENESIS

The Deoxyribonucleic Acids of Normal and Malignant Tissues

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INTRODUCTION

Schultz has eloquently stated that the genetic concept of the neoplastic process is at the core of the problem: how are the processes of cell heredity changed during this event? The alternative hypotheses to the genetic one are all examples of the genetic hypothesis specified in terms of a particular phenotype, or reduce to special cases of a genetic interpretation of the neoplastic phenomenon, or they depend upon distinctions which may eventually turn out to be artificial (Schultz, 1958). The genetic concept of carcinogenesis and tumor progression is already supported by considerable cytological evidence (Ford and Mole, 1958; Hsu, 1959; Chu, Sanford, and Earle, 1958; Levan and Biesele, 1958). This provides a strong incentive to the biochemist to investigate the chemistry of the material which presumably confers hereditary specificity, that is, deoxyribonucleic acid (DNA). Can it be shown by chemical methods that the DNA's of primary and of transplanted tumors differ from those of normal tissues? Can a relation be established between chromosomal imbalance, "abnormal DNA," and an abnormal metabolism?

The Structure of DNA

The Watson-Crick Model

It is probable that the DNA of cells constitutes a heterogeneous mixture (Bendich, Pahl, and Beiser, 1956) of linear, unbranched molecules (Gulland, 1947; Todd, 1954). On the basis of x-ray diffraction studies of DNA from a variety of sources (Wilkins, 1956; Langridge *et al.*, 1957), Watson and Crick (Crick, 1954, 1957) constructed DNA models and made the now classic proposal that DNA consists of two helical polynucleotide chains which are twined round one another. The two chains are held together by hydrogen bonds between the bases, each base being joined to a companion base on the other chain. The pairing of bases is specific, adenine going with thymine and guanine with cytosine. The phosphate groups are accessible to hydrogen or hydroxyl ions and to dyes, and are therefore on the outside, whereas the bases occur opposite one another on the inside. The guanine amino group is free. Prominent 3.36 Å spacings along the fiber axis were interpreted as due to a succession of flat nucleotides standing out perpendicular to the fiber axis to form a relatively rigid structure and consistent with the high density of DNA.

The concept of DNA as a stiff-coiled macromolecule is also based upon physical-chemical data (Jordan, 1955; Dekker and Schachman, 1954) which suggest particles having a molecular weight of 10^6 to 10^7, an axial ratio of 300:1, a diameter of about 500 mμ, and a thickness of about 2.5 mμ. This view gains additional support from direct observations with the electron microscope (Hall and Litt, 1958), which show the molecules to be threadlike and stiff, with a thickness of 1.5 to 2.0 mμ.

Two-Stranded Molecule

There is considerable supporting evidence for the two-stranded model of DNA (Crick, 1957). This includes: (a) titration curves which suggest that the bases are hydrogen bonded (Jordan, 1955; Gulland and Jordan, 1947; Shack and Thompsett, 1953, Shack, Jenkins, and Thompsett, 1953), (b) the shape and size of the molecule in solution obtained from a combination of light scattering, viscosity, and sedimentation measurements, which show that DNA in solution is highly extended but not completely straight, and that its diameter is compatible with the double helix model, (c) degradation studies with x-ray, acid, or enzymes (Thomas, 1956, Thomas and Doty, 1956) which are consistent with there being two strands

in DNA so that the molecules do not come apart until there are breaks in both backbones opposite one another; (d) chemical analysis of the molar ratios of purine and pyrimidine bases (Chargaff, 1951, 1955, 1957); (e) experiments on the effect of heat on the physical-chemical properties (Dekker and Schachman, 1954; Thomas, 1954), ultraviolet absorption spectra (Lawley, 1956), chromatography (Bendich, Pahl, Korngold, Rosenkranz, and Fresco, 1958a), and biological activity of DNA (Zamenhof, 1957); and (f) isotopic experiments dealing with the replication of the DNA of bacteria (Meselson and Stahl, 1958). A number of these points will be elaborated further.

Purine and Pyrimidine Composition of DNA

DNA molecules may be grouped by composition into two main types, an "A-T" type having adenine and thymine as the major bases, and "GC" type, in which cytosine and guanine preponderate. Animal DNA's, including those of tumors, are all of the "A-T" type, while both types occur in microorganisms and some insect viruses (Wyatt, 1952) (Table 1). Chargaff has observed a remarkable constancy in the ratio of adenine (A) to thymine (T), guanine (G) to cytosine (C) plus 5-methyl cytosine (MeC), purines to pyrimidines, and 6-amino groups to 6-keto groups (Chargaff, 1955). These ratios are in all cases nearly equal to unity. The ratio of $\frac{A + T}{G + C + MeC}$, however, gives a figure which, along with the relative 5-methyl cytosine content, distinguishes one nucleic acid from another. The base composition of DNA is constant for different tissues of the same species, but differences exist between species (Chargaff, 1951, 1957). DNA's fractionated by the method of Chargaff (Chargaff, 1955) vary in the ratios of A + T/G + C from 1.07 to 1.61, but the ratios of $\frac{A}{T}$, $\frac{G}{C}$, and purines to pyrimidines remain close to unity.

The purine and pyrimidine base compositions of a number of DNA preparations from malignant tissues have been examined (Chargaff, 1955, Uzman and Desoer, 1954). Spontaneous or transplanted malignant tissues do not differ from normal tissues with respect to their elementary base composition (Table 1) The base ratios of the diploid lymphomas are similar to those of tetraploid lymphomas (tumors 6C3HED-DBA and 6C3HED-DBA to C3H).

Although the purine and pyrimidine ratios of the DNA of normal

tissues and tumors are the same, it is possible that the sequence of the bases varies along the polynucleotide chains. It is known from studies of enzymatic digests of thymic DNA that the dinucleotides of cytosine-cytosine; cytosine-thymine, cytosine-adenine, cytosine-guanine,

TABLE 1. DNA Base Ratios of Normal Tissues and Lymphomas

Tissue	Ratio of Bases Relative to A				PU	6-AM	A + T
	A	G	C	T	PYR	6-KETO	G + C
Spleen (AKR) (7)*	1.0	0.79	0.79	0.96	1.02	1.02	1.24
Spleen (C3H) (4)	1.0	0.76	0.78	0 95	1.02	1 04	1.27
Spleen (DBA) (2)	1.0	0.76	0.73	0.91	1.07	1.04	1.28
Thymus (AKR) (2)	1 0	0.85	0.81	0.94	1.06	1.01	1.17
Leukemia (10)	1.0	0 82	0 81	1.02	0 99	0.99	1.24
6C3HED (4)	1.0	0 81	0 80	1.00	1.01	0.99	1.24
E9514A (4)	1 0	0 82	0 82	1.05	0.97	0 97	1.25
6C3HED-DBA (5)	1.0	0.79	0.85	0.91	1.02	1.09	1.16
6C3HED-DBA to C3H (7)	1.0	0.79	0 87	0 98	0.97	1.06	1.19

Abbreviations: *A*, adenine; *G*, guanine; *C*, cytosine; *T*, thymine; *PU*, purine, *PYR*, pyrimidine; *6-AM*, adenine plus cytosine; *6-KETO*, guanine plus thymine.

* Numbers in parenthesis indicate the number of determinations performed in quadruplicate

and guanine-methylcytosine occur (Sinsheimer, 1957). There is evidence for polypyrimidine (and therefore polypurine sequences) as well as for pyrimidines flanked by two purines Recently, Shapiro and Chargaff (1957a, 1957b, 1957c) and Burton and Petersen (1957) have introduced chemical procedures for classifying DNA's on the basis of: (1) relative abundance of "solitary" pyrimidine units (i.e , *purine-pyrimidine-purine-any base*) and (2) relative "polypyrimidine" density The procedure provides a useful tool for distinguishing DNA preparations even if they are indistinguishable by other means. Their results permit the conclusion that the detailed aspects of the arrangement of pyrimidine nucleotides (and therefore of purine nucleotides) vary widely in DNA specimens of different origin. At least 70 per cent of the DNA pyrimidines may occur as oligonucleotide tracts containing three or more pyrimidines in a row. The application of these methods to the characterization of DNA from neoplastic tissues will be awaited with interest.

Effect of Heat on Ultraviolet Absorption Spectra and Chromatography of Nucleic Acids

When dilute neutral solutions of DNA in water or buffer are briefly heated between 80° to 100°C, profound changes in the structure of the molecules take place (Dekker and Schachman, 1954; Shooter, 1957; Rice and Doty, 1957; Thomas, 1954). Although the magnitude of the effect may vary with the concentration of DNA or salt in the solution, a large reduction in viscosity, loss of biological activity of transforming factor, and, in some cases, lowering of the sedimentation constant (Butler and Shooter, 1957) and a reduction of the radius of gyration (Rice and Doty, 1957) have been noted. Electron micrographs indicate that the DNA helices may dissociate and coil up into amorphous patches, although a few apparently unaltered molecules survive the heat treatment (Hall and Litt, 1958). Since it is unlikely that such treatment breaks phosphodiester bonds, it is probable that there is simultaneous rupture of many weak bonds, such as hydrogen bonds. Lawley (1956) observed that DNA solutions which have been heated and then cooled to room temperature show an increase of about 30 per cent in the ultraviolet absorption spectrum. This effect may be illustrated with the samples of tumor and spleen DNA which were used in the present experiments (Table 2). In the presence of sodium ions, there is no change in the shape of the absorption curves, but the extinction coefficient increases at the wavelengths, 230 to 280 mμ. A composite absorption curve, calculated from the individual absorption curves of the nucleotides and the composition of DNA, shows a greater absorption than does DNA as commonly isolated As the difference between the so-called "theoretical" curve and the observed curve largely vanishes after heat treatment, it may be inferred that the organization of the nucleotides in the "native" DNA in some way causes a reduction in the absorptive capacity of the various purine and pyrimidine rings. This reduction might be due to π electron interaction between "stacked" purine and pyrimidine rings *in the* "native" highly organized DNA or to changes in resonance within the rings as a result of the specific hydrogen bonding between rings (Dekker and Schachman, 1954; Thomas, 1954) Thus, Lawley has suggested that the heat denaturation involves: (a) the breaking of the hydrogen bonds between pairs of bases and the separation of *sections* of the two polynucleotide chains, and (b) a rearrangement of the more flexible single chains to configurations in which the purine and pyrimidine rings are no longer

TABLE 2. Effect of Heat* on Nucleic Acid Absorption in Ultraviolet

Tissue	Per Cent of Control Value DNA	RNA
Ehrlich	128	115
Lettré-Ehrlich	137	111
Lettré-Ehrlich (Sucrose-Citrate Extract)		109
6C3HED-DBA to C3H	130	98
6C3HED-DBA	.	113
6C3HED	134	
E9514A	135	
Leukemia #10	135	
Spleen	128	

* Heated 1 hour at 100°C at pH 7, 0.01 M or 0.05 M NaCl, 0.005% solution of nucleic acid and then cooled to room temperature. Optical density read between 300 and 240 mμ with Beckman DK-2 Spectrophotometer.

superimposed (Lawley, 1956). These conclusions are consistent with recent observations that heated *Escherichia coli* DNA is reduced in molecular weight to approximately half that of the unheated molecules (Meselson and Stahl, 1958).

Interesting observations which have a bearing on the two-stranded DNA model have been made on the chromatographic properties of heated solutions of DNA (Bendich, Pahl, Rosenkranz, and Rosoff, 1958b). A DNA solution which has been heated in water for 15 minutes at 100° C may be readily eluted from DEAE-cellulose anion exchangers by weak saline solutions (Fig. 1). This is in marked contrast to the undenatured DNA, which requires concentrated salt solutions and an alkaline pH for the elution of the polynucleotides.

The relative ease of elution of DNA mixtures from anion exchangers is in part a function of the molecular weight or state of aggregation of the molecules (Bendich *et al.*, 1955, 1956, 1958a, 1958b; Rosoff *et al.*, 1957). Monodeoxyribonucleotides are completely eluted with 0.01 M phosphate (pH 7), devoid of NaCl; whereas a deoxyribonuclease digest of DNA, containing a large proportion of oligonucleotides, required increases in the NaCl concentration up to 0.22 M for quantitative elution. Fractions of nucleic acids obtained with eluents of increasing ionic strength and increasing pH have been found to show increasing sedimentation coeffi-

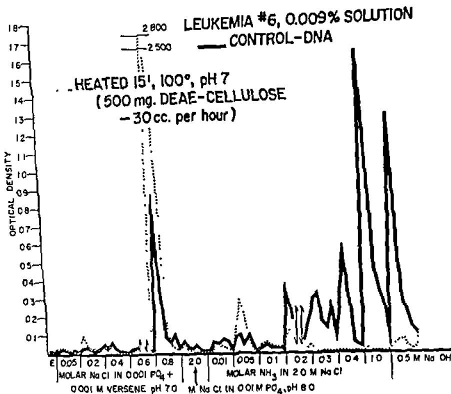


Figure 1 Effect of heat on chromatography of Leukemia #6 DNA on DEAE-Cellulose Approximately 3 mg DNA applied to each column Recovery of DNA from column control, 111 per cent, heated sample, 105 per cent.

cients (Bendich, Pahl, Korngold, Rosenkranz, and Fresco, 1958a; Rosoff, di Mayorca, and Bendich, 1957). The elution profiles of nucleic acids from different sources differ significantly. As the sedimentation constant (Bradley and Rich, 1956) or the intrinsic viscosity (Rosoff, di Mayorca, and Bendich, 1957) of the preparation increases, the elution profiles shift toward higher salt concentrations. It may be concluded that the chromatographic studies suggest that DNA molecules of diminished molecular weight are generated as a result of the cycle of heating and cooling.

Chromatography of Normal Tissue and Tumor DNA

It should be emphasized that the DNA of tissues is extremely heterogeneous (Bendich *et al.*, 1956; Butler and Shooter, 1957).

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6C3HED	134	
E9514A	135	
Leukemia #10	135	
Spleen	128	

* Heated 1 hour at 100°C at pH 7, 0.01 M or 0.05 M NaCl, 0.005% solution of nucleic acid and then cooled to room temperature. Optical density read between 300 and 240 mμ with Beckman DK-2 Spectrophotometer.

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gested the possibility that a greater per cent of DNA of the normal tissues than of the transplanted tumors was eluted by neutral saline solutions in the early peaks (Kit and Gross, 1959).

The chromatographic differences between DNA's from normal tissues and tumors could be interpreted as due to intrinsic differences in the molecular size distribution of the DNA molecules or, alternately, as due to various artifacts arising from the method of preparation or the conditions of chromatography (Bradley and Rich, 1956). The percentage of a given sample of DNA which is eluted from an anion exchanger by neutral saline solutions may be modified by any of the following factors: (a) partial degradation or denaturation during preparation, (b) contamination by protein, (c) selective loss of lower molecular weight material during preparation (Kit and Gross, 1959) In addition, contaminating RNA would be measured as DNA since the elution of DNA was followed by spectrophotometric measurements in the ultraviolet The effects of these factors on the chromatographic profiles were investigated and are briefly discussed below. DNA preparations are probably subject to some degradation by deoxyribonuclease during the isolation of the nucleoprotein from tissues (Shooter and Butler, 1956; Dounce, O'Connell, and Monty, 1957; Shack and Thompsett, 1953). Conceivably, the marked differences in the chromatographic profiles of rat brain and rat kidney reported by Bendich, Pahl, and Beiser (1956) might be attributable to the fact that the nucleases are more active in the latter tissue (Greenstein, Carter, and Chalkley, 1947). The method of Kirby (1957), which was employed in these experiments, involves the use of p-aminosalicylate and 45 per cent phenol for the extraction of DNA from the tissues. It has the advantage that it is not necessary to isolate cell nuclei, a procedure that must involve some losses, and that deoxyribonucleases are probably inactivated by the phenol To further reduce the problem of enzymatic degradation, some DNA samples were extracted from the tissues at ice-box temperatures. The chromatographic profiles of the latter preparations did not differ greatly from that of the DNA prepared at room temperature (Fig 3) The overall recovery from the columns of DNA prepared at room temperature was not significantly different from that of DNA prepared at ice-box temperature.

The manner in which the tissue is homogenized affects the chromatographic properties of the DNA When a Virtis Company Omnimixer is used to blend the tissue samples, the DNA is partly degraded or denatured and as much as 30 per cent of the subsequently isolated

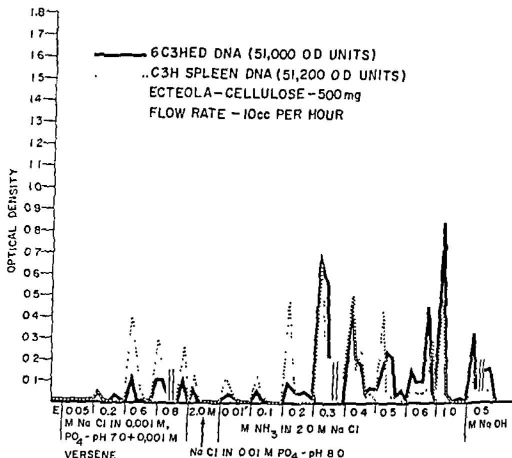


Figure 2 DNA chromatographic profiles of tumor 6C3HED and C3H spleen (ECTEOLA-CELLULOSE) Recovery: 109 per cent and 107 per cent of total optical density units applied to columns. 5 cc aliquots were collected, 30 cc per eluting solution except for the 0.8 M NaCl, the 2 M NaCl–0.3 M NH_3, and the 0.5 M NaOH solutions where a total of 60 cc was collected. The flow rates of the latter solutions and of the 0.6 M NaCl, 2.0 M NaCl–0.2 M NH_3, and the 2.0 M NaCl–1 M NH_3 solutions were reduced to about 5 cc per hour

Efforts to fractionate the DNA mixtures chromatographically have been attended with some success (Bendich *et al.*, 1955, 1958a; Rosoff *et al.*, 1957). The chromatography of both DNA and RNA from spontaneous leukemias, transplanted lymphomas, spleen or thymus, and various other tumors has been studied in this laboratory (Figs. 2, 6, and 7). The nucleic acids were isolated essentially as described by Kirby (1956, 1957, 1958). The general features of the chromatographic profiles are shown in Figure 2. Preliminary experiments sug-

sedimentation coefficients after chymotrypsin treatment, which may be due to a decrease in the size of the particles, such as might result from the breaking of intermolecular cross links (Butler, Phillips, and Shooter, 1957). Protein is present in the Kirby-DNA preparations, the amount depending upon whether the ion used in conjunction with the phenol is p-aminosalicylate, benzoate, or trichloroacetate (Kirby, 1958). However, the chromatographic profiles were not greatly modified when the DNA was subjected to chymotrypsin treatment prior to chromatography. Likewise, the profiles were essentially the same whether phenol and p-aminosalicylate, phenol and benzo-

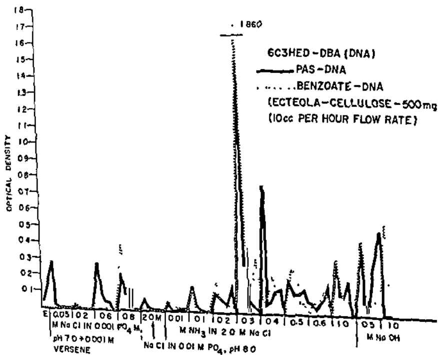


Figure 4 Chromatography of 6C3HED-DBA-80 DNA prepared by extraction of tissues at 4°C with (a) 12 volumes of 6 per cent p-aminosalicylate and 12 volumes of 90 per cent phenol containing 10 per cent m-cresol, or (b) by extraction with 12 volumes of 0.15 M sodium benzoate in place of p-aminosalicylate. The DNA was purified as described by Kirby (1957) except that the precipitated DNA was redissolved in 0.01 M NaCl instead of distilled water. Recovery of DNA: 104 per cent and 79 per cent, respectively.

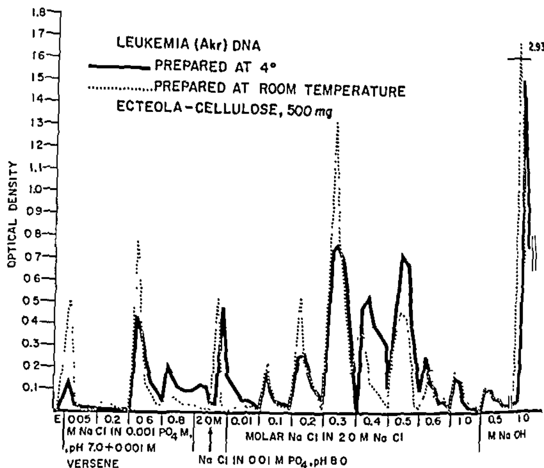


Figure 3 Chromatographic profiles of Leukemic DNA prepared by phenol-p-aminosalicylate extraction at room temperature or at 4°C. Recovery of DNA 101 per cent and 102 per cent, respectively. 5 cc. aliquots were collected, 30 cc. per eluting solution

DNA may be eluted from the ECTEOLA Cellulose columns by 0.6 M NaCl. *In contrast, only about 5 to 10 per cent is eluted by 0.6 M* NaCl when the tissue sample is homogenized with an all glass Potter-type homogenizer. The DNA chromatographic profiles of spleen samples homogenized with the glass homogenizer resembled more closely those of the transplanted ascites lymphomas. Moreover, when the ascites lymphomas were blended in the Virtis Omnimixer, the per cent of the subsequently isolated DNA eluted by 0.6 M NaCl was increased to 29 per cent.

Small amounts of residual protein markedly affect the physical properties of DNA. *In some cases, there is observed a decrease of the*

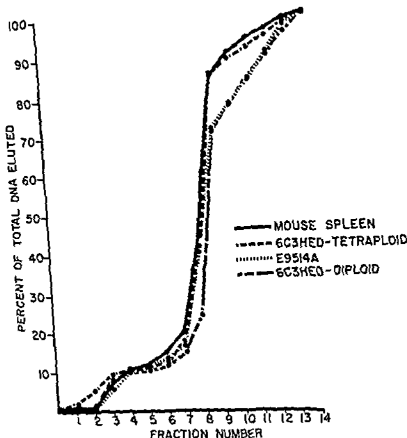


Figure 6 DNA chromatographic elution profiles on 500 mg ECTEOLA-Cellulose (Brown Co, Berlin, N H, exchange capacity, 0.39 meq per gram) of normal C3H mouse spleen, diploid lymphomas E9514A and 6C3HED, and tetraploid lymphoma 6C3HED Approximately 55–75,000 optical density units (258–260 mμ) were applied to each column Elution rate approximately 5–10 cc per hour Fraction numbers 1–14 correspond to the following eluents: (1) 0 05 M NaCl, (2) 0 2 M NaCl, (3) 0 6 M NaCl, (4) 0 8 M NaCl, (5) 2 0 M NaCl, 0 01 M phosphate buffer, pH 8, (6) 0 01 M NH_3, 2 0 M NaCl, (7) 0 1 M NH_3, 2 0 M NaCl, (8) 0 2 M NH_3, 2 0 M NaCl, (9) 0 3 M NH_3, 2 0 M NaCl, (10) 0 4 M NH_3, 2 0 M NaCl, (11) 0 5 M NH_3, 2 0 M NaCl, (12) 0 6 M NH_3, 2 0 M NaCl, (13) 1 0 M NH_3, 2 0 M NaCl, (14) 0 5 M NaOH Eluents 1–5 were dissolved in 0 001 M phosphate buffer, pH 7 With each eluent, a total volume of 30 cc was collected in six tubes, except for eluents 4, 9, and 14, in which case a total volume of 60 cc, was collected in twelve tubes

ate, or phenol- and trichloroacetate were used in the preparation of the ascites tumor DNA (Fig. 4).

DNA prepared by the Kirby procedure contains only traces of ribose. When present, most of the ribonucleic acid can be eluted from cellulose anion exchangers by 2.0 M NaCl at pH 7 (Fig. 5). It was important, therefore, to investigate whether the early 0.6 M NaCl peak of the leukemic DNA's was contaminated with RNA. It was shown by chemical analysis using the Burton modification of the diphenylamine reaction that the ultraviolet-absorbing material of this peak could be accounted for quantitatively as DNA. On the basis of the orcinol color reaction, no RNA was found.

When the preparation and the chromatography of DNA samples were very carefully controlled, the chromatographic profiles of all

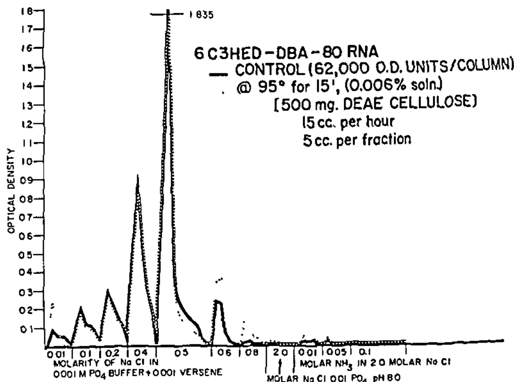


Figure 5 Effect on the chromatography of heating 6C3HED-DBA-80 RNA to 100°C for 15 minutes in 0 01 M NaCl, 0 001 M phosphate, 0 001 M versene, and then cooling to room temperature Approximately 3 mg RNA were applied to each column Recovery of RNA control, 75 per cent, heated sample, 78 per cent. A total of 30 cc was collected for each eluting solution except for the 0 5 M NaCl and 2 0 M NaCl–0 1 M NH_3 where 60 cc were collected and the flow rate was reduced to 5 cc per hour

Polli and associates (Polli, 1958; Polli and Shooter, 1958) who have noted differences in the physical-chemical properties and chromatographic profiles of the DNA preparations of normal and leukemic leucocytes from human patients. Differences between the electrometric titration curves and the effect of urea and alkali on the viscosity of the DNA solutions were found. The average sedimentation coefficients were higher than normal for DNA prepared from lymphatic leukemic cells, but somewhat lower than normal in the case of myeloid leukemic DNA preparations. The shapes of the sedimentation coefficient distribution curves did not vary greatly, and were interpreted as suggesting that the samples differed in molecular weight rather than shape (Polli and Shooter, 1958). Our results are, however, in agreement with the studies of Smith and Kaplan (1959) on the DNA of leukemic mouse tissues. The results of the present experiments and those of Smith and Kaplan do not, of course, preclude the existence of differences between the DNA of normal tissues and tumors; they merely indicate that such differences are below the level of resolution of the methods employed.

Properties of Ribonucleic Acid (RNA)

Structural Features

Present concepts suggest that RNA mediates the transfer of genetic information between the hereditary components of the cell and the cell enzymes and proteins It therefore becomes necessary to evaluate available data on the properties of RNA Although ribonucleic acids from many sources have been examined by physical methods within the last few years, no clear-cut and consistent picture of the configurational properties has materialized X-ray diagrams of RNA resemble those of DNA, but the poor quality of the diagrams has precluded any decision as to whether the similarity was accidental or whether the RNA was also a two-stranded molecule (Watson, 1957; Rich, 1957) Most studies of RNA have been complicated by spontaneous changes of molecular weight, aggregation under some conditions and degradation under others. RNA's have been prepared with molecular weights ranging from 6,000 to 290,000, according to the source, method of preparation, and method of measurement. The low values probably reflect degradation (Jordan, 1955; Timasheff, Brown, Colter, and Davies, 1958; Colter and Brown, 1956). The molecular weight of at least some RNA molecules is probably much greater Highly polymerized RNA of a molecular weight of about

samples were very similar (Figs. 6 and 7). No significant differences were observed between the DNA of normal tissues, spontaneous tumors, or transplanted tumors. This is in contrast to the results of

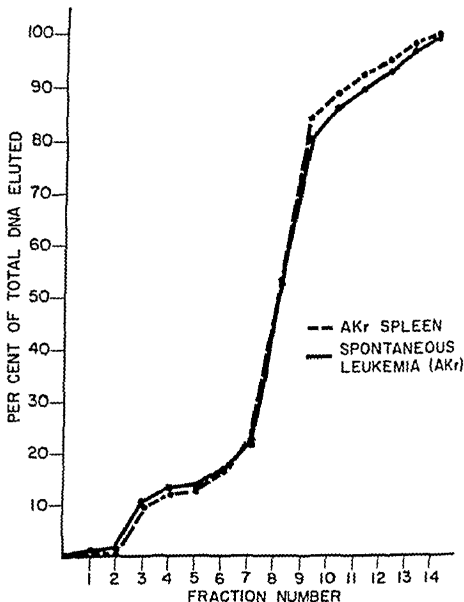


Figure 7 DNA chromatographic elution profiles on 500 mg ECTEOLA-Cellulose (Brown Co, Berlin, N H, exchange capacity, 0 39 meq per gram) of normal AKR mouse spleen and spontaneous leukemia of AKR mice. Other conditions were the same as shown in the legend to Figure 6

ley and Rich, 1956). The elution profiles of the RNA from different sources differ significantly. Preparations of tumor RNA have been chromatographed after the samples were heated in neutral salt solution for 15 minutes at 100° C and cooled to room temperature. Again unlike the DNA preparations, the chromatographic profile of the heated RNA is unchanged from that of the control samples (Fig. 5)

Possible Mechanism for the Initial Transfer of the Genetic Code from DNA to RNA

The regularities of composition observed with DNA do not obtain in the case of RNA. No equality of purines to pyrimidines, adenine to thymine, or guanine to cytosine has been observed. However, one regularity has been found for RNA from various sources The number of nitrogenous constituents carrying a 6-amino group was close, and on the average equal to that of the 6-keto compounds (Elson and Chargaff, 1955). The material analyzed included whole tissues and various subcellular fractions derived from liver and kidney, from sea urchin eggs and embryos, and from several microorganisms. It should be pointed out that among the plant viruses, there appear to be at least two cases in which the total 6-amino do not equal the 6-keto bases; namely, turnip yellow mosaic virus and potato virus.

If one assumes that DNA acts as a template for the formation of RNA which relays information to the protein polypeptide chain by another template mechanism, there should be a simple relation between the composition of RNA and DNA, since there are only four commonly occurring base groups in each nucleic acid The fact that this is not so is probably due to two factors: (1) only a fraction of the nucleic acids in the cell functions as a template and therefore only this fraction would be pertinent when comparing base ratios of the two nucleic acids; or (2) there are several different templates involved in the transfer of information from DNA to the polypeptide chains, and, as a result, the general problem of information transfer might be more easily solved by consideration of each template and its immediate chemical product (Zubay, 1958).

Assuming that nuclear RNA is the one most likely to be produced directly on the DNA because of its close cytological connection, Zubay has proposed a code in which a given nucleotide base pair of the double-stranded DNA molecule functions as a location for *one* RNA base group in a *single* RNA chain, the sequence of base groups in the latter being determined by the sequence of base pairs in the

2×10^6 has recently been prepared by Gierer (1958) from tobacco mosaic virus; while Timasheff *et al.* (1958) have isolated an RNA from Ehrlich ascites cells having a molecular weight of about 1.2×10^6. Electron micrographs showed elongated particles in both cross section and length, with dimensions of 4 to 7 mμ and 80 to 200 mμ, respectively. A high molecular weight sample has also been prepared from calf liver microsomes by Hall and Doty (1958). The low intrinsic viscosity of these RNA preparations (about 0.27 to 0.35) is noteworthy. On the basis of electron microscope studies (Hart, 1958), measurements of sedimentation, viscosity, and kinetics of action of RNase, it has been inferred that tobacco mosaic virus RNA is a *linear, single-stranded molecule* (Commoner, Shearer, and Strode, 1958; Gierer, 1958). The microsomal liver RNA of Hall and Doty (1958) manifests a linear relation of the log of the sedimentation constant or intrinsic viscosity when plotted against the log of the molecular weight This type of dependence is associated with homologous samples of linear, randomly coiled polymer chains. Provided that RNA is a randomly coiled *single chain*, one would expect the relatively tight coiling to give way to a much more expanded coil in the absence of electrolyte. This would be recognized by a much higher viscosity upon dilution with water. This is observed for RNA, clearly indicating the progressive expansion of the molecule as its counter ions become further removed from it (Hall and Doty, 1958). Upon the addition of salt, the viscosity returns to a low value, showing the reversibility of the effect. Thus, the solution properties of RNA are found to be satisfactorily correlated with a randomly coiled *single chain structure.*

The effect of heat on the ultraviolet absorption spectra and chromatographic properties of RNA is in marked contradistinction to that of DNA (Hall and Doty, 1958). RNA samples prepared at icebox temperature and precipitated from dilute salt solution by modifications of the phenol method of Kirby (1956) manifest an increase in the ultraviolet absorption spectra of about 25 per cent when the polynucleotide is heated in neutral salt solution to a temperature of 100° C. In contrast to DNA, however, the absorption is reduced to approximately the control values when the samples are cooled to room temperature (Table 2). The first heating cycle of freshly prepared RNA shows the same results as successive cycles, in contrast with DNA, where the optical density never returns to the original value after the first heating (Hall and Doty, 1958).

RNA has been fractionated on cellulose-anion exchangers (Brad-

Table 4 Ratio of Metabolism to DNA of Diploid and Tetraploid Cells

Tissue	Chromosomes	γ DNA / 10^6 cells	γ dry weight / 10^6 cells	γ histone / γ DNA	Transaminase activity*	$\mu l\ CO_2$ per hr / γ DNA	$\mu l\ O_2$ per hr. / γ DNA
Ehrlich	76	18.0	510	3.46	40.5	1.31	0.32
Lettré-Ehrlich	46	11.1	268	2.86	45.2	1.27	0.34
6C3HED-DBA-2	76–78	15.3	300	0.98	29.3	1.02	0.17
6C3HED-DBA-2 to C3H	74–79	14.2		0.90		0.99	0.18
6C3HED	40	9.8	128	0.83	21.8	1.09	0.19
E9514A		7.9	106	0.84	25.8	0.94	0.17

* Micromoles aspartate formed per hour per γ DNA (glutamic + oxalacetic → aspartic + α-ketoglutaric).

former acid (Zubay, 1958). Different binding sites are proposed with a given nucleotide base pair, depending upon which polynucleotide chain the individual members are situated. Thus, an A-T I base pair, with adenine on strand 1 and thymine on strand 2 of DNA, is visualized as binding the guanine of RNA, while an A-T II base pair, with thymine on strand 1 and adenine on strand 2, binds cytosine. This code predicts a necessary relation between the base composition of the two nucleic acids such that $\frac{A+T}{G+C}$ of DNA equals the $\frac{G+C}{A+U}$ of RNA. The correspondence of the ratio of $\frac{G+C}{A+U}$ of nuclear RNA, with that of the ratio of $\frac{A+T}{G+C}$ of DNA, is illustrated in Table 3. The purine-to-pyrimidine ratio of RNA will be one only if the two DNA chains contain the same relative amounts of 6-amino and 6-keto groups. Since the ratio of 6-amino to 6-keto is about one in the RNA samples, one may infer that the relative purine to pyrimidine content of each of the DNA strands is one (Table 3).

TABLE 3. Relation of Base Ratios of RNA to DNA

Source of Nucleic Acid	Nuclear RNA Pu/pyr	Nuclear RNA $\frac{G+U}{A+C}$	Nuclear RNA $\frac{G+C}{A+U}$	DNA $\frac{A+T}{G+C}$	Cytoplasmic RNA $\frac{G+C}{A+U}$
Rat Liver	0 86	1.00	1 27	1 33	1 59
Ox Liver	0 83	1.01	1 25	1 25	1 59
Ox Kidney	0 83	0.94	1 27	1 30	1 52
Starfish Oocyte	1 38	1.04	1.74	1 78	1 42

(*After Zubay, 1958*)

Abbreviations: *A*, adenine; *G*, guanine, *C*, cytosine, *U*, uracil, *T*, thymine, *PU*, purine, *PYR*, pyrimidine; *DNA*, deoxyribonucleic acid; *RNA*, ribonucleic acid.

The prototype of the possible interaction between the double-stranded DNA molecule and a single-stranded RNA has been observed in the case of synthetic polynucleotides. There is evidence that polyadenylic acid may interact with two molecules of polyuridylic or polyinosinic acid to form three-stranded structures (Watson, 1957;

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Rich, 1957, 1958; Felsenfeld and Rich, 1957; Morgan and Bear, 1958).

QUANTITATIVE RELATION BETWEEN DNA AND CELL METABOLISM

The frequent occurrence of aneuploidy, polyploidy, and heteroploidy in populations of neoplastic cells raises the question as to the effect of supernumerary chromosomes on cell metabolism. In order to relate the abnormal metabolism of neoplastic cells (Kit and Griffin, 1958) to the abnormal chromosome complex, the concept was put forward that a quantitative relationship exists between gene number and the primary products of gene action, provided that the cytoplasmic milieu is held constant and the genotypic milieu is changed only with respect to gene dosage (Kit *et al.*, in press). An interesting, though not ideal, system was available to examine this concept, namely, that of diploid and tetraploid ascites lymphomas and carcinomas. It was observed that DNA content parallels chromosome number in these cells (Table 4). The tetraploid cells are approximately twice as large in volume and weight as the diploid cells, although it is important to note that the diploid carcinoma cells are of the same size as the tetraploid lymphoma cells.

Respiration, anaerobic glycolysis, and transaminase activity are also ploidy dependent in the histologically related cells (Table 4). Thus, the ratio of the former metabolic parameters to DNA is approximately constant for the diploid and tetraploid lymphomas or carcinomas, although the metabolic activity of the carcinomas exceeds that of the lymphomas (Kit, Fiscus, Graham, and Gross, 1959; Kit and Gross, in press). The DNA content per cell or the ratio of metabolism to DNA was not changed as a result of the follow-

TABLE 5 Effect of Environmental Temperature of Tumor-Bearing Mice on *in vitro* Tumor Glycolysis

	Temperature		
Tissue	6°	24°	38°
E9514A	1.10*	0.95	0.90
6C3HED	1.17	1.03	
6C3HED-DBA-2	1.08	1.08	1.06
Ehrlich		1.53	1.61

* Values shown are microliters CO_2 produced per hour per gamma DNA

ing modifications of the tumor-host environment: (a) the sex of the tumor-bearing animal, (b) temperature-stress-induced modifications of the host (Table 5), (c) the number of days (between six and eleven) that the tumor cells had been grown in the host (Table 6), and (d) changes of the mouse strain used for tumor cell growth (Table 4).

TABLE 6 Effect of Duration of Growth in Host on *in vitro* Glycolysis of *Tumor Cells*

	Days in Host		
Tissue	6	8	11
Ehrlich	1 25*	1 16	1 18
Lettré-Ehrlich	1 28	1 39	1 16

* Values represent microliters CO_2 produced per hour per γ DNA.

The ratio of transaminase activity or histone content to DNA was constant for the histologically related tumors (Table 4). These results lend support to the postulated relationship. However, as discussed in detail elsewhere (Kit, Fiscus, Graham, and Gross, 1959; Kit and Gross, in press), the tumor cell populations employed in the above studies are heterogeneous, and the polyploid populations definitely do not represent euploid multiples of the basic number of chromosomes of the species of origin Moreover, the DNA charge of a particular chromosome need not be directly proportional to gene dosage The possibility of hypo- or hypermorphic gene alleles, or suppressor and modifier genes, must also be considered in evaluating experimental observations Hence, exceptions to the constancy of the ratios of metabolism to DNA are to be anticipated and, indeed, have been observed. For example, the ratio of glutathione to DNA is approximately six times as great in the tetraploid lymphomas as in the diploid lymphomas.

Observations bearing on the relationship of gene dosage to metabolism have been made by other investigators. A dependence of metabolism on ploidy has been found in haploid to tetraploid yeast strains (Ogur, 1954; Ogur *et al*, 1952) and for a variety of genetically related plant species in which gene dosage was varied (Jorgensen and Geissman, 1955). The effects of homozygosity and heterozygosity on

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Quantitative Relation Between DNA and Cell Metabolism

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6C3HED-DBA-2	1 08	1 08	1 06
Ehrlich		1 53	1.61

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* Values shown are microliters CO_2 produced per hour per gamma DNA

The above data represent a bridge between the postulated abnormal chromatin content and the abnormal metabolism of tumor cells.

ACKNOWLEDGMENTS

The experimental work reported in this paper was supported in part by grants from the American Cancer Society (P-35), The Leukemia Society, Incorporated, and the National Cancer Institute (C-4238).

The assistance of Arthur Gross, Frank Broo, Henry Szwartz, and Jane Fiscus is gratefully acknowledged

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biochemical phenotypes have been studied in yeast (Kossikov, 1957) and in humans (Singer *et al.*, 1957).

These results place restrictions on speculative models dealing with the mechanism of transfer of genetic information from hereditary units to mobile enzyme-forming systems. By implication, only one enzyme-forming template should be produced when the gene is present in haploid dosage, two when in diploid dosage, and four when in tetraploid dosage. The model proposed by Zubay (1958) in which a single RNA strand is formed on a double-stranded DNA template is consistent with the above concepts. A model in which two complementary RNA strands are formed on the DNA template, however, would lead to enzyme-forming templates having two strands, a condition contraindicated by the available observations on the configurational properties of RNA. Alternatively, to be reconciled with the data presented in the present paper, the model would require that two complementary RNA templates be formed per gene, or that other theoretical assumptions be made.

Summary

The elementary purine and pyrimidine composition, physical-chemical properties in solution, and chromatographic profiles on anion exchange resins of DNA and RNA from normal tissues and tumors have been presented. These data and other observations on the effect of heat on the nucleic acids suggest that DNA consists of a two-stranded structure of complementary intertwined helical chains, while RNA represents a single-stranded polymer.

The purine and pyrimidine base ratios of DNA samples from normal tissues do not differ from those of tumors. The chromatographic profiles of DNA prepared from transplanted lymphomas, spontaneous leukemias, and of normal spleen are also very similar. Further studies are therefore required in connection with the hypothesis that the DNA's of normal tissues and tumors are different in structure or composition.

The Zubay model for the possible mechanism of the initial transfer of the genetic code from DNA to RNA was presented. This model was related to the concept that a quantitative relation exists between the DNA, chromosome content, and cell metabolism of diploid and tetraploid cells. Data were presented indicating that the ratio of DNA to respiration, glycolysis, transaminase activity, and histone content is approximately constant for histologically related cells.

The above data represent a bridge between the postulated abnormal chromatin content and the abnormal metabolism of tumor cells.

ACKNOWLEDGMENTS

The experimental work reported in this paper was supported in part by grants from the American Cancer Society (P-35), The Leukemia Society, Incorporated, and the National Cancer Institute (C-4238).

The assistance of Arthur Gross, Frank Broo, Henry Szwartz, and Jane Fiscus is gratefully acknowledged

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Nucleic Acid Studies of Mammalian Tumor–Inducing Agents

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Numerous attempts have been made in recent years to characterize the nature of the mammary tumor-inducing agent, one of the three factors known to play a part in the origin of at least some mammary tumors of mice (Dmochowski, 1957).

It has been suggested that the agent is comparable with a cytoplasmic particle which may have arisen by transformation of a normal cell constituent, or is comparable with agents arising by mutation of cytoplasmic determinants, plasmagenes, or with the cytoplasmic factor "Kappa" of paramecia (Dmochowski, 1953).

For a number of years, we have been engaged in attempts at characterization of the agent by means of biological, biophysical, and biochemical methods (Dmochowski and Passey, 1952; Dmochowski, 1956, 1958)

A study of material which would serve as the best source of the agent for attempts at its characterization demonstrated that fresh milk of agent-harboring mice is a better source of the agent than lyophilized milk or fresh or lyophilized tumor tissue (Dmochowski, 1956). In the milk of agent-carrying mice, most of the tumor-inducing activity was found in the nonlipid fraction. The activity of this fraction was comparatively little affected by treatment with trypsin, chymotrypsin, chelating agents, and also enzymes which are known to produce the release of free phosphates, such as prostate phosphatase and snake venom diesterase (Dmochowski, 1956).

It should be added that lactating breast tissue of agent-containing mice has also been found to be a good source of the agent, although a direct comparison of its tumor-inducing activity with that of milk has not been carried out (Dmochowski and Haagensen, 1955).

As another approach to the study of the nature of the mammary tumor-inducing agent of mice, an extensive electron microscope study was carried out on ultrathin sections of mammary tumors and also of lactating breast tissue of agent-carrying and agent-free mice of a number of strains (Dmochowski, 1954). The study revealed the presence of characteristic viruslike particles within and outside the cytoplasm of mammary cancer cells of the majority of agent-harboring mice, and in the cells of some mammary tumors of supposedly agent-free mice. The characteristic particles varied in size from 500 Å to 1450 Å, with an average of approximately 900 Å diameter, and their internal zone varied in size from 250 Å to 700 Å (Dmochowski, Haagensen, and Moore, 1955; Dmochowski, 1956; Dmochowski and Grey, 1957). The appearance of these particles is shown in Figures 1 and 2.

In the lactating breast tissue of tumor-free mice, both with and without the agent, spherical and spheroid osmiophilic particles of 250 Å to 2800 Å in size were demonstrated. These particles, however, showed no internal structure seen in particles present in the sections of tumor tissue, and have been interpreted to be secretory casein and lipoprotein particles of milk (Dmochowski, 1956). The appearance of these particles is shown in Figures 3, 4, 5, and 6. Similar confirmatory results have been obtained by French (Bernhard, Bauer, Guérin, and Oberling, 1955; Bernhard, Guérin, and Oberling, 1956), and American workers (Bang, Vellisto, and Libert, 1956; Bang, Andervont, and Vellisto, 1956; Lasfargues, Murray, and Moore, 1958; DeOme *et al.*, 1959).

A study of the correlation between the results of biological tests for tumor-inducing activity and the results of electron microscope examination for the presence of particles gave agreement for both methods in the majority of agent-harboring tumors but only in 73 per cent of the apparently virus-free tumors. Although both methods of testing—that is, bioassays and electron microscopy—may not be sufficiently accurate to permit complete agreement, nevertheless a conclusion was reached that the case for regarding the viruslike particles as the mammary tumor-inducing virus has not been established in a convincing manner (Dmochowski and Grey, 1957).

If it is assumed that the Bittner agent is a virus because of its

many properties which indicate that it behaves like a virus, and if it is further assumed that the characteristic particles in tumor tissue are the agent itself, then the question arises of why these particles are not found in the lactating breast tissue of agent-carrying mice which has been found to have considerable tumor-inducing activity. Similar questions may, however, also be raised when it is assumed that they are particles of a contaminating virus unconnected with the origin of breast cancer in mice. Perhaps we should, as Dr. Andervont pointed out some time ago, refer to this virus as the "elusive cancer virus" or the "clever virus" hiding behind its hormonal and genetic companions of mischief (Andervont, 1957).

This agent, as is well known, may remain inactive or "quiescent," to use Dr. Andervont's term, for many months. This, indeed, has been brought forward by some as an argument against its viral nature (Dmochowski, 1953). However, there are ordinary or infectious viruses, as is well known, which behave in a similar manner. The term "latent" or "latency" has been purposely avoided in view of the many ways the term is used. The fact remains that in the lactating breast tissue examined, no structures resembling virus particles could be found, although extracts of this tissue show high tumor-inducing activity. It would be of interest to examine other organs, such as spleen or lungs, for the presence of the characteristic particles, as these organs have also been shown to possess tumor-inducing activity. This activity, however, is lower than that shown by the extracts of lactating breast tissue of agent-carrying mice (Dmochowski, 1953). For this reason, our attention has been centered on lactating breast tissue and its final product, milk.

The availability of mice, genetically identical, but differing by the presence or absence of the agent, made it possible to set up mouse dairies for the purpose of getting small but adequate quantities of mouse milk for studies of the mammary tumor-inducing agent. Dairies of mice of the following strains have been set up: Strain A, agent-carrying; Strain Af, genetically identical but agent-free; and RIII, agent-harboring strain. It should be mentioned that strain Af mice have been obtained by foster-nursing strain A mice by C57 Black agent-free females. Every possible precaution was taken to prevent the possibility of newborn strain A mice ingesting even a drop of their own mothers' milk. Nevertheless, after three generations of inbreeding, two mice developed mammary cancer. The progeny of one of them was kept and in due course in succeeding generations has shown a high incidence of breast cancer. This strain of mice is

now in its 16th generation of inbreeding, and mice of this line do occasionally develop mammary cancer. Altogether, 9 out of 252 females which have lived to the earliest tumor appearance, that is, 358 days, have developed mammary tumors. This is an incidence of 3.6 per cent. The tumors developed at a late age of between 400 to 600 days. Care has been taken to eliminate the progeny of such mice from the strain, and for the past two years, no mammary tumors have been observed in mice of this subline. In the Af strain dairy, only one female has, so far, been observed to develop breast cancer.

Chemical and Physical Studies

After a number of trial experiments in which the methods of ultracentrifugation and electrophoresis of milk were used, a biological study of the various fractions of milk, obtained by these two methods combined, was carried out. The fractions were tested in $(C57 \times Af)F_1$ hybrid mice in order to determine their tumor-inducing activity following each treatment of the original sample of milk obtained from the dairy of agent-carrying strain A mice.

The bioassays were carried out on strain A milk treated in the following manner: The milk was first defatted by centrifugation at $600 \times g$ for 20 minutes. After removal of fat, the remaining fraction was decaseinated by treatment with chymotrypsin (0.2 mg./ml.) at 38° C for five minutes. The coagulated milk was then centrifuged at $600 \times g$ for 20 minutes and the resulting supernatant was centrifuged at $105{,}000 \times g$ for two hours at 4° C. The small pellet from high-speed centrifugation was resuspended in citrate buffer of ionic strength 0.01 at pH 6.0 to the original volume of the sample of milk. Part of the resuspended pellet was used for bioassays and the rest for *zone electrophoresis*.

The electrophoresis was carried out at 4° C in a trough with powdered cellulose in citrate buffer with a current of 450 V and 15 mA. After a period of 20 hours, the cellulose block was cut in half-inch segments, the buffer removed by filtration through Whatman No. 1 paper, and aliquots analyzed for phosphorus (King, 1932) and for protein by the method of Lowry *et al.* (1951). The results of the chemical analysis are shown in Figure 7. On the basis of the Folin-Lowry curve, the segments were pooled into three fractions for bioassays in $(C57 \times Af)F_1$ test mice. As can be seen in Figure 7, the major portion of material was taken in the fraction from around the origin, and the two fractions toward the anode and cathode showed only small quantities of phosphorus and protein.

Most of the protein and more than 65 per cent of the phosphorus have been found in the central segment or fraction II. It should be pointed out that the main purpose of this experiment was not to show what resolution of components in the pellet from A strain milk could be obtained, but simply to find out whether the mammary tumor-inducing virus could be subjected to this technique and recovered in satisfactory quantities.

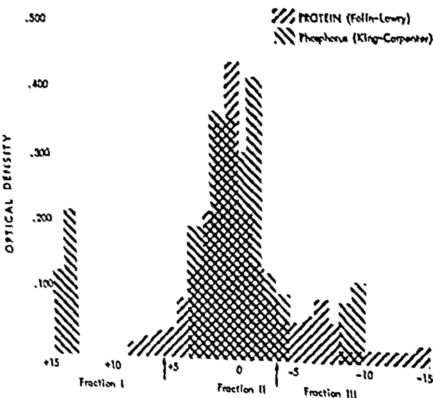


Figure 7 Electrophoretic pattern of high speed centrifugal pellet from strain A milk

The results of bioassays of the various fractions of milk obtained, after 18 months of observation, are shown in Table 1. As can be seen, treatment with chymotrypsin resulted in a decrease in the tumor-inducing activity compared with the defatted milk, but the high-speed centrifugal pellet from trypsinized milk still retained a high tumor-inducing activity. The activity of the supernatant may be the

now in its 16th generation of inbreeding, and mice of this line do occasionally develop mammary cancer. Altogether, 9 out of 252 females which have lived to the earliest tumor appearance, that is, 358 days, have developed mammary tumors. This is an incidence of 3.6 per cent. The tumors developed at a late age of between 400 to 600 days. Care has been taken to eliminate the progeny of such mice from the strain, and for the past two years, no mammary tumors have been observed in mice of this subline. In the Af strain dairy, only one female has, so far, been observed to develop breast cancer.

Chemical and Physical Studies

After a number of trial experiments in which the methods of ultracentrifugation and electrophoresis of milk were used, a biological study of the various fractions of milk, obtained by these two methods combined, was carried out. The fractions were tested in (C57 × Af)F_1 hybrid mice in order to determine their tumor-inducing activity following each treatment of the original sample of milk obtained from the dairy of agent-carrying strain A mice.

The bioassays were carried out on strain A milk treated in the following manner: The milk was first defatted by centrifugation at 600 × g for 20 minutes. After removal of fat, the remaining fraction was decaseinated by treatment with chymotrypsin (0.2 mg./ml.) at 38° C for five minutes. The coagulated milk was then centrifuged at 600 × g for 20 minutes and the resulting supernatant was centrifuged at 105,000 × g for two hours at 4° C. The small pellet from high-speed centrifugation was resuspended in citrate buffer of ionic strength 0.01 at pH 6.0 to the original volume of the sample of milk. Part of the resuspended pellet was used for bioassays and the rest for zone electrophoresis.

The electrophoresis was carried out at 4° C in a trough with powdered cellulose in citrate buffer with a current of 450 V and 15 mA. After a period of 20 hours, the cellulose block was cut in half-inch segments, the buffer removed by filtration through Whatman No. 1 paper, and aliquots analyzed for phosphorus (King, 1932) and for protein by the method of Lowry *et al.* (1951). The results of the chemical analysis are shown in Figure 7. On the basis of the Folin-Lowry curve, the segments were pooled into three fractions for bioassays in (C57 × Af)F_1 test mice. As can be seen in Figure 7, the major portion of material was taken in the fraction from around the origin, and the two fractions toward the anode and cathode showed only small quantities of phosphorus and protein.

the basis of the results of this experiment, it may be concluded that the active tumor-inducing particle does not have a large net charge at pH 6.0.

It should be added that the pellet from Af strain milk treated in a

TABLE 2 Bioassays of Electrophoretic Fractions from High-speed Centrifugal Pellet (105,000 × g — 2 Hours) of Strain A Milk in (C57 × Af) F_1 Mice

NUMERATORS / DENOMINATORS = MICE WITH MAMMARY TUMORS / MICE ALIVE AT EARLIEST TUMOR APPEARANCE

DURATION OF BIOASSAYS - 18 MONTHS

TYPE OF FRACTION TESTED	DILUTIONS		
	10^{-2}	10^{-3}	10^{-4}
FRACTION I Anode Side	0/19 -- --	0/18 -- --	0/19 -- --
FRACTION II Origin Area	18/20 95% 12M	8/17 47% 11M	1/18 6% 18M
FRACTION III Cathode Side	0/15 -- --	0/20 -- --	0/20 -- --

similar manner has given essentially the same pattern, but the electrophoretic analysis was not carried out under conditions which would give maximum resolution.

In view of the results of bioassays and electrophoresis obtained with the high-speed centrifugal pellet from milk of strain A agent-carrying mice, electron microscope studies have been carried out on sections of the high-speed centrifugal pellet of defatted and decaseinated strain A milk, which was found to be composed of an outer, pale ring and an inner, more dense, brownish zone. Similar studies were carried out on high-speed centrifugal pellets of strain RIII agent-harboring and strain Af agent-free, defatted and decaseinated milk. These studies revealed the presence of characteristic viruslike particles in the outer ring of pellets from both RIII (Figs. 8 and 9) and A strain milk (Figs 10 and 11), similar in appearance, although

result of resuspension of some of the particulate material before or during the removal of the supernatant.

TABLE 1. Bioassays of Various Fractions of High-mammary-cancer Strain A Milk in (C57 × Af) F_1 Mice

NUMERATORS / DENOMINATORS = MICE WITH MAMMARY TUMORS / MICE ALIVE AT EARLIEST TUMOR APPEARANCE

DURATION OF BIOASSAYS - 18 MONTHS

TYPE OF FRACTION TESTED (TREATMENT)	DILUTIONS		
	10^{-2}	10^{-3}	10^{-4}
DEFATTED MILK (600 x g for 20 min.)	16/20 80% 12M	12/16 75% 10M	13/18 72% 10M
WHEY (Incubation with chymotrypsin)	4/19 21% 13M	12/20 60% 12M	2/15 13% 13M
SUPERNATE (10^{-1}) (105,000 x g for 2 hours)	12/19 63% 12M	--	--
PELLET (105,000 x g for 2 hours)	9/20 45% 10M	9/19 47% 14M	11/22 50% 10M

The results of bioassays of the electrophoretic fractions from the high-speed centrifugal pellet of strain A milk, with tumor-inducing activity demonstrated in the previous table, are shown in Table 2. Only one fraction, that is, fraction II in the origin area, has shown tumor-inducing activity. Extensive migration of the tumor-inducing material has not occurred after 20 hours of electrophoresis under the described conditions. It appears that it has been possible in the present experiment to avoid contamination of other fragments by using zone electrophoresis in a small glass trough. Previous trials carried out by using a column for electrophoresis had shown tumor-inducing activity in varying amounts in all fractions in the column (Dmochowski, 1956). Thus, it has been possible to localize the tumor-inducing activity more specifically in the electrophoretic pattern. On

of RNA content in the pellet may arise from losses during removal of the supernate from the pellet (viz., the high activity of the supernate indicated in Table 1) and also from slight variations in dilution of the milk with small quantities of saline used to moisten the gland during milking.

It should be pointed out that although DNA, as estimated by the Burton-Dische reaction (Burton, 1956), has been found to be present in small quantities in mouse milk (1.8 micrograms/ml.), assays of the high-speed centrifugal pellets by the same method failed to reveal any DNA Thus, the biologically active fraction of milk treated in the described manner has been found to contain RNA but no detectable DNA. In order to check the validity of the orcinol reaction, ultraviolet absorption readings of the pellet material were made by using a Beckman DK2 recording spectrograph. The absorption spectra were those one would obtain from nucleic acids and they agreed quantitatively with the results of the orcinol reaction.

After the presence of RNA in the pellet was ascertained, the effect of ribonuclease digestion of the high-speed centrifugal pellet of defatted and decaseinated strain A milk was studied. Nucleic acid determinations were made before and after ribonuclease treatment The resuspended pellet was incubated for 30 minutes at 20° C with

TABLE 3 Ribonucleic Acid Analyses of High-speed Centrifugal Pellet (105,000 × g − 2 Hours) of Strain A Milk before and after Ribonuclease Treatment

MATERIAL (FRACTION)	AMOUNT OF RIBONUCLEIC ACID/10 ml.		
	ACID SOLUBLE (Micrograms)	ACID INSOLUBLE (Micrograms)	TOTAL
ORIGINAL PELLET (I)	103	131	234
PELLET AFTER RIBONUCLEASE TREATMENT (II)	20	10	30
SUPERNATE FROM (II) (105,000 x g)	25	154	179

somewhat smaller than those present in sections of mammary tumor cells. While the average diameter of viruslike particles in sections of high-speed centrifugal pellets of milk was found to be approximately 700 A, the average diameter of particles of similar appearance present in mammary tumor cells was calculated to be approximately 900 A. It is not known whether the observed difference in size, assuming the particles present in milk and tumor tissue are the same, is the result of treatment to which the milk has been subjected.

Characteristic viruslike particles of size similar to that of particles present in A and RIII strain milk have also been found in the outer ring of the pellets from milk of strain Af apparently agent-free mice, but only in small quantities and after prolonged search (Figs. 12 and 13). There appears to be a difference, but only quantitative, between the electron microscope appearance of this part of pellets from milk of genetically identical agent-carrying strain A and of agent-free Af mice. Whether this difference has any connection with the high mammary tumor incidence in strain A mice, and the low incidence of breast cancer in Af mice, requires further investigation.

The electron microscope appearance of the inner dense zone of the high-speed centrifugal pellets from milk of all three strains of mice has been found to be similar. This zone contained only amorphous material as shown in Figure 14.

After it was ascertained by means of bioassays that the agent is present in a pellet obtained by centrifugation at 105,000 × g of defatted and decaseinated milk, biochemical studies were carried out for the presence of RNA and DNA in similarly prepared pellets from milk of strain A mice. These studies led to the first successful colorimetric determination of RNA, because of the absence of the substance (possibly lactose) which interfered with all previous attempts at RNA estimation in mouse milk. However, it should be pointed out that the interfering substance is not always removed by high-speed centrifugation. A modified orcinol method of ribonucleic acid determination (Hurlbert, Schmitz, Brumm, and Potter, 1954) was used to facilitate reading micro-amounts of ribonucleic acid found in the pellet. The estimated amount of RNA obtained by this method in one experiment was found to be approximately 23 micrograms per each milliliter of strain A agent-carrying milk. However, other determinations gave much lower amounts of RNA, and indicate that a comparison of total RNA in high-speed centrifugal pellets of milk from A and Af strain mice may be invalidated by the wide variation in RNA content in different samples of mouse milk. This variation



Figure 3 Electron micrograph of cytoplasm of lactating mammary tissue of strain A mouse. Numerous secretory particles are seen ×15,000.



Figure 4 Secretory particles in strain A lactating mammary tissue at higher magnification. These particles show no internal structure. ×21,000.



Figure 1 Electron micrograph of a limited field of cytoplasm of an RIII strain mammary tumor cell. Characteristic viruslike particles may be seen ×40,000

Figure 2 Electron micrograph of a duct with adjoining cytoplasm of a strain A mammary tumor cell. Characteristic particles are present. Microvilli extending from the cytoplasm are seen to be extruding particles. ×10,000.



Figure 8 *Electron micrograph of a section of outer zone of high-speed (105,000 × g, 2 hours) centrifugal pellet from defatted and decaseinated agent-carrying RIII strain milk* Characteristic viruslike particles are present ×24,000

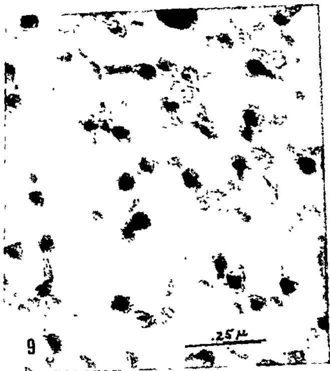


Figure 9 The same at higher magnification ×80,000.

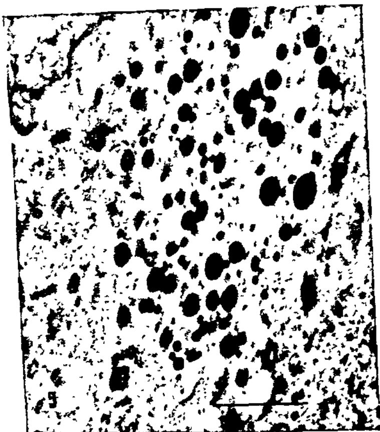


Figure 5 Electron micrograph of cytoplasm of an RIII strain lactating mammary tissue. Secretory particles with no internal structure are present. ×21,000.

Figure 6 Electron micrograph of cytoplasm of an RIII strain lactating mammary tissue. Mitochondria and endoplasmic reticulum may be seen. ×25,000.

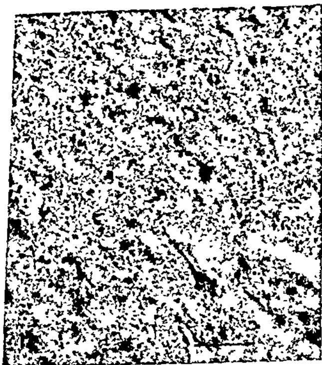


Figure 12 Electron micrograph of a section of outer zone of high-speed (105,000 × g, 2 hours) centrifugal pellet from defatted, decaseinated strain Af, agent-free, milk. Some characteristic particles may be seen ×15,000



Figure 13 Selected field of the outer zone of high-speed (105,000 × g, 2 hours) centrifugal pellet from Af strain milk. Particles with characteristic internal structure may be seen. ×73,000.

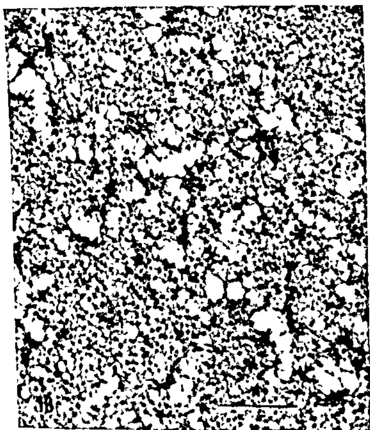


Figure 10 Electron micrograph of a section of outer zone of high-speed (105,000 × g, 2 hours) centrifugal pellet from defatted, decaseinated agent-carrying strain A milk. Particles with characteristic internal structure are seen. ×18,000.

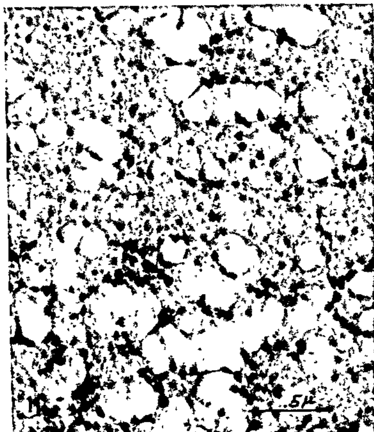


Figure 11 The same at higher magnification ×36,000.

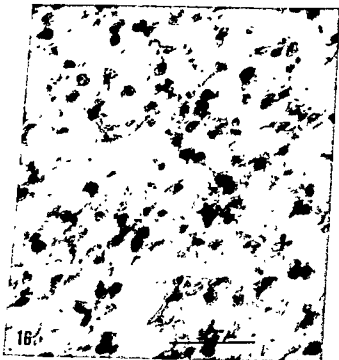


Figure 16 Electron micrograph of part of Genetron-treated RIII strain milk pellet Particles with characteristic structure may be seen ×43,500

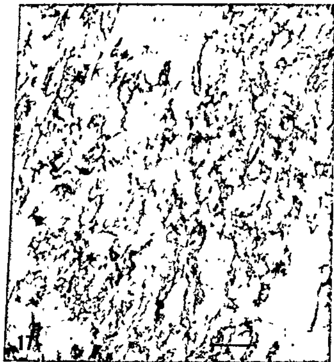


Figure 17 Electron micrograph of a section of material present at the interphase between the aqueous and fluorocarbon layers Only amorphous material present. ×12,000.



Figure 14 Electron micrograph of inner zone of high-speed (105,000 × g, 2 hours) centrifugal pellet of RIII strain *milk*. Mostly amorphous material present. ×12,000.

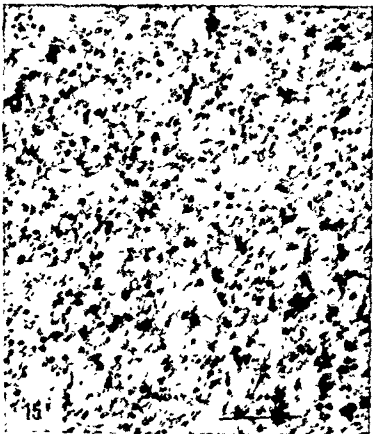


Figure 15 Electron micrograph of high-speed (105,000 × g, 2 hours) centrifugal pellet from the aqurous layer of Genetron-treated high-speed centrifugal pellet from defatted and decaseinated RIII strain *milk*. General appearance of the pellet. ×18,000.

TABLE 5 Bioassays of High-speed Centrifugal Pellets of Strain A Milk Following Ribonuclease or Ethanol Treatment in (C57 × Af)F_1 Mice

$\frac{\text{NUMERATORS}}{\text{DENOMINATORS}} = \frac{\text{MICE WITH MAMMARY CANCER}}{\text{MICE ALIVE AT EARLIEST TUMOR APPEARANCE}}$

DURATION OF EXPERIMENT	MATERIAL TESTED	DILUTIONS		
		3.2×10^{-1}	2×10^{-1}	10^{-1}
8 Months	Milk defatted decaseinated	--	--	2/15 13% 8M
	Pellet incubated	--	3/23 13% 7M	--
	Pellet incubated with ribonuclease	--	3/22 14% 6M	--
	Pellet treated with ethanol	3/25 12% 7M	--	--
7 Months	Milk defatted decaseinated	--	--	0/27 -- --
	Pellet incubated with ribonuclease	--	--	7/42 17% 6M

the high-speed centrifugal pellet with 75 per cent cold ethanol. This treatment resulted in a small, yellowish precipitate which was removed by centrifugation at 600 × g for 20 minutes The precipitate was resuspended in saline to the volume of the original milk sample and tested in (C57 × Af)F_1 mice. The results of this treatment, and also of other experiments in which ribonuclease digestion of the pellet was used, are shown in Table 5. As can be seen, the observation on ribonuclease treatment, already mentioned, has been repeated. At least some tumor-inducing activity is retained by the pellet following either digestion with ribonuclease or treatment with cold ethanol The results so far obtained indicate that precipitation with cold ethanol may be applied for the isolation of the agent.

In view of the known studies on tobacco mosaic virus and on some

10 micrograms of ribonuclease per 1 ml. The ribonuclease-treated material was then centrifuged for two hours at 105,000 × g, and the resulting pellet and supernatant tested for the presence of acid-soluble and acid-insoluble RNA and also tested for tumor-inducing activity in (C57 × Af) F_1 test mice.

The results of ribonucleic acid analysis of the high-speed centrifugal pellet from strain A agent-harboring milk before and after treatment with ribonuclease are shown in Table 3. As can be seen, the ribonuclease altered the solubility as well as the sedimentability of RNA in the original pellet. It should be noted that a small fraction of ribonucleic acid appeared to be resistant to the action of ribonuclease.

The results of bioassays on the fractions obtained from ribonuclease treatment of the high-speed centrifugal pellet from defatted strain A milk are shown in Table 4. The results, although far from complete, indicate that at least some tumor-inducing activity is retained in the pellet following treatment with ribonuclease. It is of interest that the supernatant has also shown some activity.

An attempt was also made to determine the effect of treatment of

TABLE 4 Bioassays of High-speed Centrifugal Pellets of Strain A Milk Following Ribonuclease Treatment in (C57 × Af) F_1 Mice

NUMERATORS / DENOMINATORS = MICE WITH MAMMARY CANCER / MICE ALIVE AT EARLIEST TUMOR APPEARANCE

DURATION OF EXPERIMENT	MATERIAL TESTED	DILUTIONS 10^{-1}	10^{-3}
6 Months	Defatted Milk	--	0/16 6% 6M
	Pellet Resuspended	--	3/17 18% 6M
	Pellet treated with Ribonuclease	2/19 11% 6M	2/17 12% 6M
	Supernate from Treated Pellet	1/19 5% 6M	0/19 -- --

neurotropic viruses which demonstrated activity in the nucleic acid portion (Schramm and Gierer, 1957; Fraenkel-Conrat, 1957; Colter, Bird, Moyer, and Brown, 1957), experiments were carried out in an attempt to find out whether a mammary tumor-inducing nucleic acid can be extracted from agent-carrying strain A mouse milk.

The technique of Gierer and Schramm (1956), modified by Kirby (1957), was applied to defatted strain A milk and to high-speed centrifugal pellets of this milk. Preliminary results of the bioassays of defatted milk of strain A mice, treated by this technique, are shown in Table 6.

As can be seen in Table 6, the aqueous phase obtained by phenolic extraction of milk from strain A mice has, so far, shown no tumor-inducing activity, although the control material was found to have considerable activity. It remains to be seen whether mammary tumors will develop at a later age of the test animals. The aqueous layers have been found to contain nitrogen in amounts varying from 78 to 138 micrograms per 1 ml

The fluorocarbon deproteinization technique, devised by Gessler and his associates (Gessler, Bender, and Parkinson, 1956; Porter, 1956; Gessler, 1956), was also used in an attempt at extraction of the mammary tumor-inducing agent from high-speed centrifugal pellets of A and RIII high-mammary-cancer and of Af low-mammary-cancer strain milk, following defatting and decaseinization Biological tests of the aqueous phase obtained by this method have been carried out but it is too early as yet for any results. Electron microscope examination was also carried out on sections of pellets obtained by high-speed centrifugation of the aqueous phase of fluorocarbon-treated high-speed centrifugal pellets from milk of the three different strains of mice. The results of this examination are shown in Figures 15 and 16.

The electron microscope study of sections of the sediment obtained by high-speed centrifugation of the final material in the aqueous phase appears to indicate that some purification of the characteristic viruslike particles has been achieved by this method The number of particles has materially decreased and they appear to be somewhat affected by this treatment. *The results of bioassays of the fluorocarbon*-treated pellet material from strains A and RIII milk are not yet available[1]. Correlation of the conclusions reached on the basis of appearance of these particles in the electron microscope with subse-

[1] See Addendum, p 116

TABLE 6 Bioassays of Phenol Extracted Strain A Milk in $(C57 \times Af) F_1$ Mice

$\frac{\text{NUMERATORS}}{\text{DENOMINATORS}} = \frac{\text{MICE WITH MAMMARY TUMORS}}{\text{MICE ALIVE AT EARLIEST TUMOR APPEARANCE}}$

DURATION OF BIOASSAYS	TYPE OF FRACTION TESTED	DILUTIONS				
		None	5×10^{-1}	5×10^{-2}	10^{-1}	10^{-2}
13 Months	Defatted milk	17/21 81% 10M	--	--	--	--
	Aqueous phase	*0/23* -- --	--	--	--	--
9 Months	Defatted milk	--	--	--	7/15 47% 8M	7/28 25% 7M
	Aqueous phase	--	0/35 -- --	0/39 -- --	--	--

quent results of the bioassays should prove very interesting. The electron microscope study of the denatured protein at the interphase between the aqueous and fluorocarbon layers has failed to reveal any intact particles (Fig. 17).

It should be mentioned that this method has been utilized for the purification of Rous sarcoma, vaccinia, and tobacco mosaic virus by Gessler and his associates (1956). It has recently been used with success in morphological and biological studies of vaccinia and Rous sarcoma by Epstein (1958a, b).

Turnover Studies

In view of the long waiting period for bioassays of the mammary tumor-inducing virus, experiments were carried out on the possible usefulness of turnover studies of nucleic acids in mice with regard to a shortening of the assay period. The attempt was based on the estimation of uptake of C^{14}-labeled adenine into RNA and DNA of newborn susceptible mice injected with milk of strains A agent-carrying and of Af apparently agent-free mice.

The experiments were carried out in the following manner: Litter-mate female mice of $(C57 \times Af)F_1$ hybrids, 12 to 24 hours old, were given an injection of 0.1 ml. of either strain A or Af milk, diluted 10^{-3}. After various time intervals, following the inoculation of milk, the animals received an injection of C^{14} adenine, and the total viscera of animals were analyzed for the content and radioactivity of the nucleic acids.

The results obtained in the studies on incorporation of C^{14} into RNA are shown in Table 7 As can be seen, a considerable uniformity has been found in the average amounts of RNA and in the incorporation of the isotope into RNA. No great difference was observed in the incorporation of C^{14} into RNA in mice whether in the presence or absence of the mammary tumor-inducing agent for any of the times tested, except perhaps in Experiment B.

It should be pointed out that each number within this experiment, and also the experiment shown in Table 8, is the average of determinations made on two or three pairs of animals, and was compared with simultaneous determinations on litter-mate controls. All analyses were carried out in duplicate.

The results of the studies on the incorporation of C^{14} into DNA are shown in Table 8 As can be seen, again the average amount of DNA per mouse in each experiment was rather uniform. The incorporation of C^{14} into DNA as per cent of the injected dose appeared

TABLE 7. Incorporation of C^{14} into RNA

EXPERIMENT NUMBER	TIME AFTER INJECTION OF		TYPE OF MILK INJECTED	MILLIGRAMS OF RNA PER MOUSE	C^{14} CONTENT OF RNA	
	MILK	C^{14} ADENINE			AS PER CENT OF INJECTED DOSE	SPECIFIC ACTIVITY C/min/mg.
A	1 Day	6 Hours	Af A	1.70 1.72	1.47 1.53	1613 1587
B	3 Days	1 Day	Af A	2.09 2.20	4.96 4.96	4515 4080
C	8 Days	1 Day	Af A	2.93 2.89	2.92 2.87	4187 4093

to be elevated 10 per cent to 20 per cent in mice given the agent-containing strain A milk. The difference was not always apparent when only the "specific activity" of the DNA was determined. It should be mentioned that considerable variation in the nucleic acid metabolism was found among litter-mates and especially among different litters of mice. The need for the use of larger numbers of litters per determination and for the litter-mate controls is therefore apparent.

It may be concluded that further experiments to ascertain the validity and reproducibility of these results are required before any influence of the agent on the nucleic acid metabolism can be regarded as established. Similarly, additional experiments would have to be carried out before this type of procedure could be extended to a useful assay for the presence of the agent in various dilutions and purified preparations of the agent. Should this indeed take place, a more rapid bioassay for the mammary tumor-inducing agent, based on such measurements, would then be feasible

Summary

A highly active "pellet preparation" of the mouse mammary tumor-inducing virus obtained by high-speed centrifugation of defatted, decaseinated strain A milk has been studied by zone electrophoresis The conditions employed avoided cross-contamination of fractions during processing as had been observed in earlier studies, and recovery of the mammary tumor-inducing activity was good.

"Pellet preparations" of the virus from strain A milk which had been treated with either ribonuclease or cold 70 per cent ethanol retained mammary tumor-inducing activity. These "pellet preparations" contained ribonucleic acid but no detectable deoxyribonucleic acid

Electron microscope examination of ultrathin sections of high-speed centrifugal pellets of agent-harboring defatted and decaseinated strain A milk, which have shown high tumor-inducing activity, demonstrated the presence of characteristic viruslike particles in such pellets These particles have also been observed in similarly prepared pellets from RIII agent-carrying milk, and in pellets of supposedly agent-free AI strain milk, although in considerably smaller number.

Similar "pellet preparations" obtained after deproteinization of strain A milk with either aqueous phenol or a fluorocarbon (1, 1, 2-trichloro-1,2,2-trifluoroethane) were studied by bioassay and electron microscopy.

TABLE 8 Incorporation of C^{14} into DNA

EXPERIMENT NUMBER	TIME AFTER INJECTION OF		TYPE OF MILK INJECTED	MILLIGRAMS OF DNA PER MOUSE	C^{14} CONTENT OF DNA	
	MILK	C^{14} ADENINE			AS PER CENT OF INJECTED DOSE	SPECIFIC ACTIVITY C/min/mg.
A	1 Day	6 Hours	Af A	1.26 1.39	0.78 1.01	1290 1360
B	3 Days	1 Day	Af A	2.07 2.39	3.38 3.90	2955 2950
C	8 Days	1 Day	Af A	6.12 5.87	1.74 2.30	1210 1630

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Electron microscope study of sections of pellets from high-speed centrifugation of material obtained by fluorocarbon treatment of high-speed centrifugal pellets of strain A milk revealed the presence of viruslike particles similar to those present in the milk before treatment with fluorocarbon. Bioassays of this material are being awaited.

Turnover studies with C^{14}-labeled adenine as a nucleic acid precursor indicate the mammary tumor-inducing agent may have an effect on the incorporation of radioactive adenine into nucleic acids of young mice.

Addendum

An assessment of the results of bioassays of high-speed centrifugal pellets from agent-containing strain A milk, which had been treated with fluorocarbon, has shown tumor-inducing activity in this material equal at this stage (nine months after injection) to that of the starting material (original pellet). This appears to be of interest since electron micrographs of this material have shown the presence of the characteristic particles.

ACKNOWLEDGMENTS

These investigations were supported in part by U.S Public Health Service Research Grants Nos *C-2952* and *C-3679* from the *National* Cancer Institute, National Institutes of Health, and by American Cancer Society Grant-in-Aid No E-75.

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Radiation in Relation to Cancerogenesis and Mutation: A Few Points

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The etiologic problem of radiation-induced cancers, as that of all experimental cancers, is dominated by the riddle of the "merging point." Any etiologic hypothesis concerning a given cancerogenic agent implies a hypothesis as to the nature of the malignant transformation, and as to the genotypic expression of malignancy at the level of the somatic cell. Then one must ask whether these hypotheses are compatible with conceivable etiologies for the other cancerogenic agents. If one admits that the malignant character is the same whatever the agent *responsible for cancerogenesis*, then a point exists where the diverse processes, differently sparked by the various cancerogenic agents, do merge. Therefore, in discussing radiation-induced cancers, one must keep in mind that the same types of cancers may eventually be produced by chemicals, by viruses, by hormonal upset, or by the intrusion of aberrant cellular material.

At this point, one must carefully avoid embracing local radiation-induced cancers (such as cancers of the skin) that appear at the site of local exposures, within the same etiology as both systemic (such as leukemia) and localized (such as mammary gland) cancers that follow total body irradiation.

I

The main feature of a cancer cell is its ability to divide under those conditions where normal cells stop dividing. It is not accelerated

growth; it is uncontrolled growth. The fact that normal growth is carefully controlled means that within each synthetic chain lies some repression of that chain. Since each synthetic chain is linked to at least one gene, it means that in addition to this promoting gene there is a repressing counterpart, in such a fashion that all genes go in pairs, the producer and the represser. Curbing the latter would result in the loss of control for the corresponding synthetic chain. Uncontrolled growth, therefore, can be considered in terms of damage caused to represser genes, i.e., in terms of mutations.

But the control of growth is not a cell event. It is a tissue or even an organ event, which evidently results from the summation of multiple individual cell events, the summation being made at the level of the tissue or of the organ. This implies that the repressing compounds, i.e., the products of represser genes, may diffuse from cell to cell within the tissue or the organ, and establish a certain level of concentration. Since such diffusion necessarily takes place, it follows that damage to a represser gene of a single cell is very unlikely to result in uncontrolled growth of that cell, because surrounding cells will maintain the concentration of repressing compounds at the normal level. In order to become manifest by the formation of a tumor, the mutated cell must meet special conditions which prevent this compensating process.

This concept has already been suggested by others in slightly different terms (e.g., Failla, 1958). It fits in with a number of well known facts concerning cancerogenesis, in particular:

The general toxicity of cancerogenic agents to cells (Haddow and Robinson, 1937);
The competition between two cell populations, the mutated and the normal ones (Burnet, 1959),
The so-called two-step mechanism, and the existence of so-called co-cancerogens (Berenblum and Shubik, 1947).

Regarding the latter, I wish to recall here the first case of co-cancerogenesis ever reported, since it is relevant to the next point of this paper. As early as 1929, Lacassagne and Vinzent gave one local dose of about 2000r to rabbits at the site of an inflammatory focus previously formed by the injection of bacteria. They observed that this combined treatment frequently and promptly elicited the rise of sarcomas in the irradiated zone.

This means that a critical hereditary change was induced by irradiation, and that the inflammatory tissues constituted a favorable

terrain for the rise of a malignant tumor as a result of that change. It is well known that, in the absence of such local conditions, a single local dose of 2000r does not produce malignant tumors. Conversely, chronic irradiation does, from which we may infer that chronic irradiation secures the two required steps. According to the preceding theory, these two steps would be: (*a*) a mutation consisting of the inactivation of a represser gene in a cell; (*b*) general alteration of the tissues which would prevent the supply of extraneous repressing compounds to that cell.

II

Let us focus our attention on the first step, keeping in mind that x-ray doses no higher than 2000r yield a *number of such* events.

This dose produces only 5×10^{-2} ionization per molecule of DNA (Table 1), a low figure but one which may be high enough in view of the fact that many cells are irradiated and that each of them contains many DNA molecules. But if the particular genes are considered, the probability of direct ionization becomes much lower, since the cross section of a gene probably occupies *only* a small part of a DNA *molecule* (Table 1).

TABLE 1. Absorption of 50 kv x-rays by DNA

Dose	100,000r
Number of ionizations per molecule (M W 7×10^6)	2.5
Energy imparted to the molecule	83 ev
Inactivation dose (Do, 63) for the streptomycin resistance marker in *Pneumococcus* DNA (direct effect)	4×10^6 r
Quantum yield for this inactivation	$\simeq$ 1

From Latarjet, Ephrussi, and Rebeyrotte (1959)

Thus, 4×10^6r are required to produce one ionization in that part of the DNA molecule associated with streptomycin resistance in Pneumococcus, while only 2000r have a high probability of inducing a cancer under proper conditions. This strongly suggests that the x-ray-induced mutagenic condition, which may be postulated at the origin of a tumor, does not result from direct effect, but rather from the action of a radiation-formed intermediate on the DNA. Muller (1952) has already substantiated this idea. More recently, it has been

TABLE 2. Absorption of ultraviolet light (2537 Å) by DNA

Incident dose	100,000 ergs/mm²
Number of incident photons per nucleotide	20,000
Number of absorbed photons per nucleotide	360
Energy absorbed per nucleotide	1.7 kev
Energy absorbed per molecule (M.W. 7 × 10⁶)	3.4 Mev
Inactivation dose (Do, 63) for the streptomycin resistance marker in Pneumococcus DNA	80,000 ergs/mm²
Quantum yield for this inactivation	10^{-5}

From Rebeyrotte and Latarjet (1959)

shown (Wood, 1959) that 80 per cent of the lethal effects of x-rays on yeast is due to indirect effects, and only 20 per cent is due to direct effect.

In the case of ultraviolet radiation, high doses are required to produce cancers of the skin in mice, but these doses do so in all treated animals. The need for high doses is probably due, in this case, to the low quantum yield of the ultraviolet photon on DNA (Table 2). Both the inactivation dose, for λ 2537 Å, on the /Sr marker in Pneumococcus (considered as an example of an injury to a gene) (80,000 ergs/mm²), and the cancerogenic dose in the skin of mice (of the order of 50,000 ergs/mm² at the level of the basal layer of the epidermis), are very high. This means in the latter case that whereas DNA is injured, a tremendous number of other photochemical alterations are produced in the cell. Thus, this treatment may simultaneously achieve both steps of the cancerogenic process—the mutation and that general alteration of the tissues which enables the mutated clone to show up.

III

The time has come when the biological changes induced by radiation should be correlated with precise chemical reactions in the genetic material. As an example of the lines along which we may be required to proceed, I wish to quote the particular case of peroxidation of DNA.

Weiss (1958) has shown that peroxidation of DNA by x-rays starts at the level of the nuclear double bond of the pyrimidine bases. In my laboratory, Ekert and Monier (1959) have identified the peroxides produced by x-rays on thymine in aqueous solution. Such

peroxidation opens the double bond and fixes a hydroxyl radical on one side and a hydroperoxide radical on the other one. But several isomers are formed simultaneously in different relative amounts.

This process is irreversible, contrary to the di-hydroxylation of the open double bond that ultraviolet light may produce on uridylic acid (Sinsheimer, 1954), although the peroxide is not very stable and can be decomposed by an excess of radiation In a 10^{-3} M aqueous solution, thymine peroxide is formed at the rate of 7.5 parts per 100 for an absorbed dose of 100 kilorads. Thus, 1000 rads would peroxide seven pyrimidine bases in a DNA molecule containing 20,000 nucleotides (in the very unlikely case that the rate of peroxidation would be the same in a DNA molecule).

No special importance should be attached to this reaction, at least for the time being. Many other reactions which have not yet been deciphered may be produced by irradiation of DNA. The only reason for underlining this reaction is that we know it, and therefore can describe it as an example of many possible changes.

What may be the biological significance of such a local change? It is very unlikely that it represents a "specific" chemical change endowed with particular properties For example, thymine peroxide does not compete with thymine in thymine-less *Escherichia coli* (Latarjet, unpublished). The opening of the double bond represents too large a steric alteration to leave any chance that thymine peroxide may act as an anti-pyrimidine, and therefore may be incorporated as such by the bacterium. It would be copied even less at the time of DNA *replication* But radiation produces it *in situ*; the radiation-formed peroxide lies *ipso facto* at the site of thymine, as though it had been incorporated; and this is true for all organisms, including mammals, even if they cannot incorporate pyrimidines.

It is hard to tell whether such substitution has a functional meaning, a genetic meaning, or any meaning at all. With regard to function, will such substitution modify the specificity of the synthesis which that particular DNA molecule controls? Nobody knows. Considering heredity, if one agrees that such change will not be replicated, one may surmise that it constitutes a troublesome spot for replication. At this particular locus, error is likely to occur with regard to interconnections of bases in the daughter strands, since a base will not recognize its usual sister. Such an error may show up as a change in the sequence of bases, i.e., in the coding of these daughter strands. That, in itself, constitutes a hereditary change which might be visualized as a point mutation This mutation would be a conse-

quence of peroxidation, but would have no "chemical" relationship with peroxidation as such. It would require one division cycle to show up.

IV

Lwoff, Siminovitch, and Kjeldgaard (1950; see also Lwoff, 1953) discovered that ultraviolet radiation efficiently induces viral synthesis in lysogenic bacteria. It was found thereafter that in terms of the energy required, ionizing radiations are even more efficient (Latarjet, 1951; Marcovich and Latarjet, 1958). Moreover, Jacob (1952) observed that nitrogen mustard also is an inducer. Since these three agents are cancerogenic, a tempting syllogism was offered which would bridge the gap between those cancers caused by physical and chemical agents, and those caused by viruses. In addition, the concept that cells of higher organisms could harbor proviruses which could be turned into neoplastic viruses by physical and chemical cancerogens, brought an interesting scheme for the hereditary transmission of the high susceptibility to certain spontaneous cancers. In mice, the extensive use of homozygous strains led to the discovery that at least some hereditary high susceptibilities to spontaneous cancer are associated with the presence of filtrable agents. The constancy of the high incidence through successive generations implies the vertical transmission of the agent under one form or another. Under the present state of our knowledge, we can conceive of two forms:

1. *Permanent Infection.* At all steps of its development, including the egg, the animal would harbor an infectious subcellular agent (virus). The state of infection would be maintained throughout the whole cycle of the host. In formulating this hypothesis, it must be kept in mind that the viral cycle may present some eclipse phases during which no active material can be extracted from the broken cells. But the cells remain infected in the sense that they still constitute infective centers capable of propagating the infectivity. Gross (1956) has supported this idea in the case of lymphoid leukemia in mice.

2. *Virogenic State.* According to the model provided by lysogenic bacteria (see Wollman, pp. 43 to 59, this volume) the agent would be transmitted in the form of a provirus. The hereditary material which conveys the genetic information of the virus would be integrated within that of the cell in a noninfectious form which would divide synchronously with the cell. The provirus would per-

petuate itself among some somatic cells of the animal until certain endogenous or external circumstances caused it to become an active virus. In particular, cancerogenic agents could account for this induction.

The idea that lysogeny might come into play in the field of cancer viruses has already been evoked in several theoretical discussions (e.g., Stanley, 1958). However, if such discussions are to be placed on the more solid ground of facts, there are apparently only five experimental works which can be taken into consideration at the present. To recall them briefly:

1. Radiological differences exist between the temperate bacteriophages—those which are capable of lysogenization—and the virulent ones—which cannot lysogenize. A comparison of the temperate phage λ and the virulent phage T2, shows that both have about the same size (but not the same amount of DNA), and both are active on the bacterium *E. coli* K12S. Phage λ is several times more resistant than phage T2 to ultraviolet radiation, and slightly more resistant to x-rays. In other words, T2 contains more radiation-sensitive material than λ. Conversely, the capacity of the host cell K12S to produce T2 is several times more resistant to radiation than its capacity to produce λ. The bacterium contains some radiation-sensitive material which is necessary to produce λ, but which TS production does not need. It looks as though the virulent virus contains some radiation-sensitive material required for virus production, which the temperate virus does not carry and therefore must find in its host (Garen and Zinder, 1955; Latarjet, 1956).

Rubin and Temin (1959) have compared the radiological behaviors of the Rous sarcoma virus (R) and of the Newcastle disease virus (N) either free or grown on chicken fibroblasts They found a situation similar to the preceding one, where N would correspond to T2, and R to λ. Free R is about ten times more resistant than N to ultraviolet radiation, but the capacity of chicken fibroblasts to produce N is several times more resistant to radiation than is their capacity to produce R. According to this picture the Rous sarcoma virus appears to be of the temperate type, in contrast to the Newcastle virus. This would mean that the genome of the Rous virus can "lysogenize," i.e., can be integrated with that of its host cell as long as no viral production proceeds.

2. The second experiment, like the three that follow, deals with mouse leukemia In the Ak inbred strain, about 75 per cent of the animals spontaneously develop a lymphoid leukemia which is associ-

ated with the presence of a filtrable agent (Gross, 1951). This agent can transfer the disease to homologous strains.

In a large-scale experiment we have checked whether or not the newborn Ak mouse is immune to superinfection with the Gross virus (Rudali, Duplan, and Latarjet, 1957). This work was undertaken because cellular immunity is a straight consequence of lysogenization (Lwoff, 1953). The establishment of a prophage in a cell confers complete immunity on that cell toward the corresponding free phage.

Newborn Ak mice (less than one day of age) received a cell-free extract from Ak leukemic tissues by subcutaneous injection. Controls received a cell-free extract from nonleukemic tissues of strain XVII. In all, more than 2000 newborn animals were treated. As a result of the injection of the leukemic extract, the frequency of leukemia rose from 75 per cent to 90 per cent, and the latency of the disease was shortened by a factor of two. The first cases appeared after about eighty days, instead of after six months; the maximum frequency was reached at four and a half months, instead of nine months. No effect was observed in the controls. Moreover, the incidence of leukemia was normal among the progeny of the superinfected animals which had developed an early disease. According to this experiment, the young Ak mouse is sensitive to the leukemogenic activity of the agent.

3. Since virus multiplication is efficiently induced by x-rays in lysogenic bacteria, we wondered if a somewhat similar phenomenon would take place in newborn Ak mice. Would total body x-irradiation accelerate the leukemic process, and increase its frequency? Another large-scale experiment has been carried out along this line (Reverdy, Rudali, Duplan, and Latarjet, 1958). A total of 1527 Ak mice, less than one day of age, were divided into seven groups. Of these, 420 served as nonirradiated controls; the others underwent a total body exposure to 180 kv x-rays with doses of 50, 100, 200, 250, 350 and 500r. Leukemias were recorded over a period of 500 days. The results (Table 3) can be summarized as follows:

a) Doses smaller than 500r did not significantly change the latency, but slightly decreased the frequency. This decrease was more pronounced in males than in females, and was more pronounced with the greater doses. Since this change was contrary to what could have been expected from an induction of the lysogenic type, we wondered if such an induction could have been masked by some other effect of the radiation. In particular, x-rays are known to produce an involution of the thymus, a target organ in lymphoid leukemia.

TABLE 3. Leukemogenesis among Ak mice which received one total-body x-irradiation at birth

Dose of x rays (r)	Survival at 90 days (%)	Incidence of leukemia among survivors (%)		Average latency of leukemia (days)	
		males	females	males	females
0	68	63	82	292	274
50	56	45	84	250	273
100	40	45	77	280	275
200	44	53	74	276	283
250	34	29	71	230	225
350	21	26	58	249	226
500	8	33	56	120	186

b) Consequently, 606 other newborn Ak mice received a single dose of 250r on total body, the thymus being protected, or a non-irradiated isologous thymus being grafted after irradiation. In both cases, the same decrease of the incidence of leukemia was observed. Thus, this decrease is not due to involution of the thymus. This conclusion was confirmed by another experiment in which it was shown that cortisone, when given to newborn Ak mice, does not decrease the incidence of leukemia, although it produces a temporary involution of the thymus. Therefore, the decrease shown in Table 3 remains unexplained.

c) With the dose of 500r, the latency was suddenly shortened, while the trend to lower frequency ceased. A new type of leukemia, which could be called "radiation-induced," seemed to appear.

As a conclusion of this work, we can say that, under our experimental conditions, doses smaller than 500r do not produce any evidence of viral induction A dose of 500r seems to produce some new effect which could be induction, but has not yet been shown to be so. If this were so, the more general question would be opened: Are radiation-induced leukemias due to a "lysogenic induction" phenomenon?

4. Another experimental approach to this question *consists of* using a strain of mice with a very low frequency of spontaneous leukemia, of inducing the disease with radiation, and of looking for a leukemogenic filtrable agent in the leukemic tissues. This *has recently* been done by Gross (1959) in the C3H strain, and by Kaplan (1959) with Lieberman in the C57Bl strain. *In both* experiments, the

cell-free extract of radiation-induced leukemia was injected into suckling mice. Of these animals, 11 per cent (Gross) and 17 per cent (Kaplan) developed a leukemia of their own after several months. That leukemia was induced by a cell-free agent and not transferred by cells was indicated by Kaplan from the fact that an extract from C57 mice, when passed through F_1 hybrids, induced tumors of hybrid genotype. This activity appeared in irradiated thymus glands 30 to 40 days after irradiation. In Gross's experiment, the somewhat low activity of the first extract was enhanced by serial passages through C3H suckling mice, the final extract producing leukemia in 65 per cent of the treated animals.

5. Finally, I will mention an experiment on homologous transplantation, carried out in my laboratory by Duplan (1958, 1959), which shows that Ak normal cells contain the full antigen corresponding to the leukemogenic agent. As recipient, Duplan used the XVII inbred strain of the Institut du Radium of Paris, which is not histocompatible with Ak, and which has a very low incidence of spontaneous leukemia (0.2 per cent). By the age of two weeks, the XVII mouse becomes definitely insensitive to Ak material. Leukemia cannot be transferred by cells or induced by the agent from Ak to XVII young adults.

Duplan used *young XVII adult mice which had been rendered* partially tolerant to Ak material, either by the Medawar-Billingham technique or by total body x-irradiation. In the former case, 2×10^6 Ak normal spleen cells were injected into the embryos at the seventeenth day of intra-uterine life. In the latter case, a dose of 700r was given to young adults; then restoration was achieved with Ak fetal (nonleukemic) liver cells. Fifty days after these treatments, 3×10^7 Ak leukemic cells were injected intraperitoneally. Three types of positive responses appeared among these animals.

a) *Within only three months, some mice* developed a lymphoid leukemia of the Ak immunogenetic type. In these animals, tolerance to the homologous Ak cell was established successfully.

b) Among the other animals which did not accept the graft of leukemic cells, a number of lymphoid leukemias appeared, within a year or more. Immunogenetically, these belonged to the XVII recipient strain. This is the point which is relevant to our problem: These mice, which were not tolerant to Ak cells, had become tolerant to the Ak leukemic agent. According to the mechanism of tolerance, this means that the normal Ak cells used to establish this situation contain the antigen corresponding to the leukemic agent.

c) After 200 to 400 days, a few animals developed mammary carcinomas, which, according to the experimental controls, were due to the injected material—probably the polyoma virus harbored by our Ak strain. The conclusion concerning this virus is the same as in the preceding case.

The picture which comes out of these five experiments is not clear. Experiments 1 and 4 favor the idea of a lysogenic-like situation in the Rous sarcoma and in C3H and C57Bl mice. The three others seem to indicate the presence of complete virus particles in normal tissues of embryos and adults of the Ak strain. Much more work is needed before any precise concept takes shape, but the amount of experimental data gathered on this problem during the last couple of years sounds highly encouraging.

V

It has been clearly demonstrated in a number of instances that cells introduced into a foreign host may break out and release cancerogenic viruses. A few experiments have recently suggested that even simpler cellular material can be released and exert cancerogenic activity. Such an effect has been observed after the injection of nucleoproteins extracted from tumors (Paschkis, Cantarow, and Stasney, 1955; Bielka, Graffi, and Krischke, 1957), and of nucleic acid extracted from leukemic tissues (Latarjet, Rebeyrotte, and Moustacchi, 1958; Latarjet, 1959). This suggests a new kind of consideration concerning the cancerogenicity of radiations Among radiation damages to cells the leakage of cellular material must be considered. It is well known that irradiation rapidly increases the amount of nuclear components (in particular, free nucleic acid and its degradation products) which leak out of the cells. This is true in microorganisms, where these compounds are found in the suspending medium, and in mammals as well, where the compounds remain within the tissues.

So far, most people have visualized radiation-induced cancers as originating in those cells which have themselves been altered by the radiation. This concept implies *ipso facto* that the alteration is not lethal to these cells, and this is not easy to understand. Here, we now envisage that these cancers may originate in cells which have incorporated some material released by those cells which have been altered by the radiation In this case, the alteration can be—*and, in fact*, is likely to be—lethal to the cell. It must be profound enough to favor

the release of material and to change this material eventually to the extent of making it cancerogenic.

It is relevant to quote here Blum's (1958) quantitative experiments on the induction of skin cancer in mice by repeated exposures to ultraviolet radiation given at different intervals. From his results, Blum reached the conclusion that the multiplication process is irreversible and is accelerated by each exposure. Thus, a given tumor would contain several clones which originated at different times, and whose units would display different growth rates. The curve representing the increase in size of the tumors as a function of time can be extrapolated to time zero, thus giving the initial volume of the multiplying unit. In this way, Blum found $V_o = 10^{-14}$ mm^3, the volume of a large molecule. He interpreted his results as indicating that each irradiation induces a certain number of cells to release some material capable of entering other cells and of multiplying within them.

Five points have been considered in this study. How do they link to one another? This is not readily apparent. Other points should be taken into account, such as the effect of radiation on immunity, and more probably still others which we are unable to formulate or even imagine because of the lack of fundamental knowledge. For example, I have discussed mutations, infection, lysogeny, radiation effects, along classical lines. But these lines are certainly too narrow. Transfers of genetic information from cell to cell, or from virus to cell, may pass through chemical channels other than DNA alone. Spontaneous mutations may occur in the absence of cell division. Radiation chemistry within the cell is still in the prenatal stage.

Many changes can be expected in the near future with regard to concepts of this problem.

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Genetic Replication and Carcinogenesis

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Oncologists have always sought a common etiology for cancer, and we may now be approaching the time when resolution of most differences between various theories is possible. The indications are that cellular deoxyribonucleic acid (DNA) is involved in carcinogenesis, and that a common ground in etiology theory may result from studies on the replication of genetic mechanisms of cells. The bacteria and bacterial viruses have been extremely useful tools for the study of gene replication and theoretical genetics, and much of our theory regarding these areas has been gained using these systems. These studies have, at the same time, contributed much information concerning virus reproduction and associated host-parasite relationships. Recent findings in regard to bacterial transduction and lysogenic bacteria, if found to hold for animal viruses, will certainly erase the last vestiges of conflict between the somatic mutation and viral theories of cancer induction.

THEORIES OF DNA SYNTHESIS AND GENETIC REPLICATION

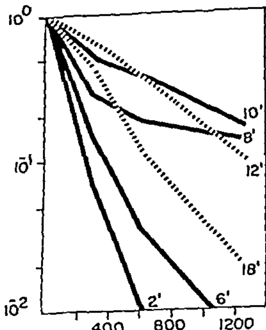
Evidence has accumulated for a number of years which indicates that the hereditary information of most cells is carried in the cellular DNA. In 1953, Watson and Crick produced a model for the molecular structure of DNA which satisfies the physical chemistry and organic chemistry data regarding DNA (Watson and Crick, 1953a). In this model the DNA molecule consisted of two polynucleotide

chains, coiled around a common axis. The two chains were held together by hydrogen bonding between pairs of the purine and pyrimidine bases of the two chains. Only certain pairs of the purine and pyrimidine bases would fit into the structure, and one member of each pair had to be a purine and the other a pyrimidine. A nucleotide which occurred on either chain would specifically determine the identity of its partner on the other chain. Tautomeric evidence indicated that adenine paired with thymine, and guanine with cytosine. The sequence of bases on the chain was thought to be irregular, and Watson and Crick believed that this sequence constituted the code responsible for carrying the genetic information. In their model, if the actual order of the bases on one of the chains was given, the exact order of the bases on the other would be known. This fact suggested to them how the DNA molecule might duplicate itself (Watson and Crick, 1953b, c). They imagined each of the strands to be a template for formation of the complementary strand. Prior to duplication the hydrogen bonds would break, the two chains unwind, and the exposed bases serve as templates for formation of another complementary chain.

The Watson-Crick model for DNA has now been accepted as probably representing quite closely the actual structure existing in DNA molecules. However, their theory as to duplication of genetic DNA does not satisfy a considerable part of recent biochemical and genetic data (see Stent, 1958, for review of this subject). We will not go into the bacteriophage recombination or bacterial transduction data here. It will be sufficient to say that experimental evidence from these areas has led to the theory of copy-choice to explain genetic recombination in bacteria and phage (Lederberg, 1954; Hershey, 1952). Copy-choice theory demands that homologous regions of two genetic templates from different parental sources mate, or synapse; and that recombination of genetic factors results because the repli-

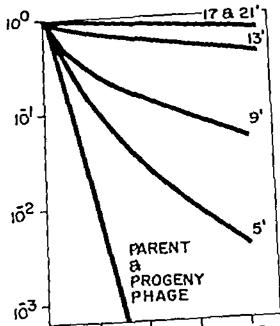
Figure 1a Inactivation of intracellular phage by ultraviolet radiation During the period of intracellular phage multiplication from two minutes to ten minutes, the intracellular vegetative phage becomes increasingly resistant to ultraviolet rays, but inactivation of infective centers does not produce a family of multiple-hit curves with the same slope as anticipated (Redrawn from Luria and Latarjet, 1947)

Figure 1b Inactivation of intracellular phage by P^{32} decay Note the similarity of curves showing P^{32} inactivation of infective centers with curves of ultraviolet inactivation of infective centers given in Figure 1a See text for experimental procedures and discussion (Redrawn from Stent, 1955)



SURVIVAL CURVES OF T^2 INFECTIVE CENTERS AT TIMES OF INCUBATION (FROM LURIA & LATARJET 1947)

Figure 1a



SURVIVAL OF B/r^*-T^{2*} INFECTIVE CENTERS AS A FUNCTION OF THE FRACTION OF THE INITIAL P^{32} ATOMS (FROM STENT 1955)

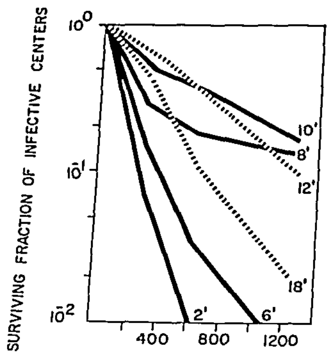
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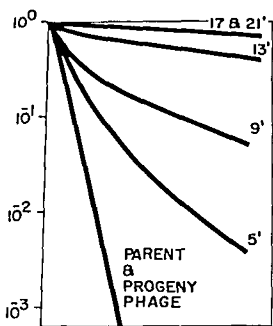
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and all of its replicas. (They would be radioactive also, since growth was taking place under conditions where the only phosphorus available was labeled.) However, the curves obtained with this experiment were very similar to those of Luria and Latarjet (Fig. 1b).

This result led Stent to theorize that those structures of the vegetative phage containing the genetic information during the eclipse period were quite different from the double-stranded helical type of DNA. He stated that if, at the time of infection, the genetic information were transferred from the phage DNA to a newly synthesized protein molecule, the greatly reduced sensitivity of the vegetative phage to ultraviolet and P^{32} decay could be explained (since protein

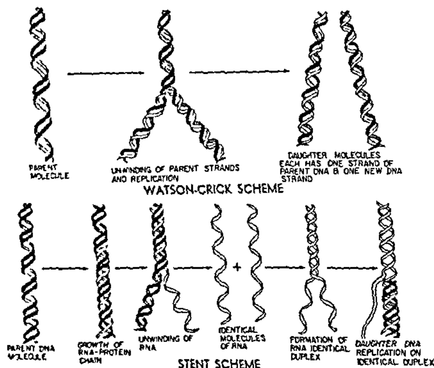


Figure 2 The Watson-Crick scheme (upper diagram) for synthesis of new genetic DNA. As the parent strands of DNA unwind, new strands of DNA are synthesized along the exposed bases of each strand. The new DNA is a facsimile of the opposite strand of parental DNA in each case, and each newly synthesized molecule of DNA contains one strand of parental DNA and one of new DNA (Redrawn from Watson and Crick, 1953b)

The Stent scheme (lower diagram) for synthesis of new genetic DNA See text for explanation and discussion (Redrawn from Stent, 1958)

cating genetic structure growing along these mated templates replicates along one member of the synapsed template pair for a while, and then switches over and replicates along the other member of the template pair. Stent (1958) has pointed out that the Watson-Crick theory for DNA replication does not allow for this mating of templates and switching back and forth of growing replicas.

Of more importance to this discussion are certain experiments which indicate that the genetic information is not always carried in the DNA alone, and that during the replication of the genetic structure it must be carried also in some transient structure involved in replication of the DNA. In 1947, Luria and Latarjet, attempting to follow the intracellular multiplication of phage by ultraviolet inactivation of intracellular phage, obtained an unexpected set of inactivation curves. They had thought it would be possible to follow intracellular phage multiplication with this technique since the ultraviolet dose needed for complete inactivation of all phage particles within a bacterium should be directly proportional to the number of intracellular phages. Therefore, curves for successive times of incubation of infected bacteria should reveal the number of active particles per bacterium at each time. In their experiments, however, the curves obtained did not allow for such a simple explanation (see Fig. 1a). The main change in the curves during the first 11 to 12 minutes of intracellular growth was a progressive increase in resistance to radiation, but the curves also showed a progressively increasing concavity which was just the opposite of that expected. Stent (1955) pointed out that this type of curve would be obtained if, during the first part of the intracellular growth period, the phage genetic information was no longer carried in the DNA, but in some other moiety of greater resistance to ultraviolet.

A different type of experiment was then performed by Stent. His experiments were designed to study the sensitivity of phage-infected bacteria to inactivation by P^{32} decay at various stages of intracellular growth. P^{32}-labeled *Escherichia coli* growing in labeled medium were infected with P^{32}-labeled T-2 phage. Phage development was allowed to proceed, and at varying times after infection samples of the infected culture were frozen and stored at $-196°$ C while P^{32} decay was allowed to proceed. At regular intervals samples were thawed and assayed for their ability to produce infective phage. Stent reasoned that multiple-hit curves would be obtained using this technique, where the Luria-Latarjet experiments had failed, since decay of incorporated P^{32} ought to inactivate the labeled parental DNA

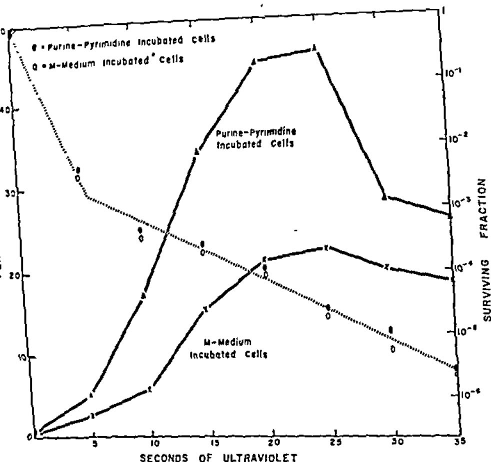


Figure 3 Ultraviolet survival and induction of nonlactose fermentation mutants in *E. coli* strain B incubated for one hour in basal medium (M-Medium) or in purine-pyrimidine-supplemented basal medium (Purine Pyrimidine Incubated Cells) immediately prior to irradiation. Immediately following irradiation the cells were plated on a complete agar medium allowing expression of both survivors and mutants. The broken line represents the survival curve of both cultures, the solid lines, the mutation frequency-radiation dose curves.

has a lower ultraviolet sensitivity and phosphorus content than DNA). As an alternative he stated that the genetic information could continue to reside in polynucleotide chains and still be represented by a definite sequence of purine and pyrimidine residues, but this chain might be tied to a protein backbone which would assure the integrity of the polynucleotide after P^{32} disintegrations had broken the polyphosphodiester bonds (Stent, 1955).

To account for the above experimental data and also for copy-choice theory, Stent proposed a modification of the Watson-Crick theory for DNA replication (Stent, 1958). The essentials of Stent's theory are given in Figure 2 along with the essential features of the Watson-Crick scheme. In his theory, double-stranded helical molecules of DNA serve as templates for synthesis of nucleoprotein molecules consisting of one ribonucleic acid strand with a protein backbone. This strand grows within the deep groove of the DNA helix and leads to a three-stranded structure. The specific base sequence of the RNA would be governed by the base pairs of the DNA double helix, since each of these can form another pair of hydrogen bonds with one and only one type of base. The building blocks for the RNA chain would be amino acid-ribonucleotide complexes, and a polypeptide backbone chain could thus grow on the outside of the DNA groove simultaneously with the growth of the polyribonucleotide chain on the inside. The new ribonucleoprotein molecule would then unwind from the DNA template by some process such as the "speedometer cable" type of rotation suggested by Levinthal and Crane (1956). After unwinding, further ribonucleoprotein molecules would be synthesized by repetition of the process.

The genetic information would have been transferred from the DNA double helix to a single ribonucleoprotein chain. The free RNA protein chains then mate with other RNA protein chains of exactly the same base sequence, and through hydrogen bonding of the identical base pairs form a two-stranded structure, which Stent calls the "Identical RNA Duplex." This straightens out the coiled RNA protein chains, exposing their purine and pyrimidine bases, so that they can act as templates for synthesis of progeny DNA molecules. The replica DNA would be synthesized by the inverse of the process by which the single RNA protein strand was formed. Only one strand of RNA acts as a template at a time, while the other remains inactive. However, DNA replication can switch back and forth from one RNA strand of the identical duplex to the other Growth of daughter DNA on the surface of the identical duplex causes separation of the two

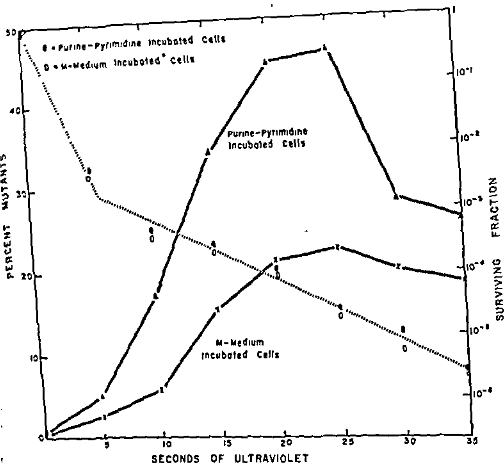


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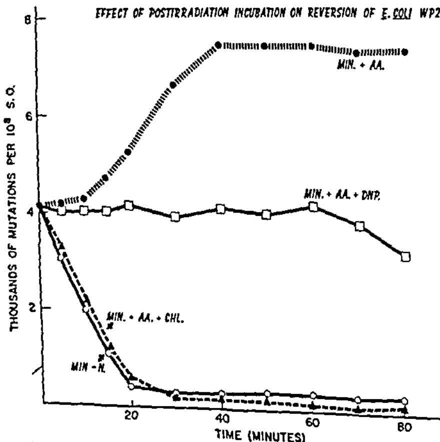


Figure 4 Effect of postirradiation incubation in different media on ultraviolet induction of reverse *mutations in E. coli* strain WP2. *Immediately* following irradiation equal aliquots of the bacterial culture were suspended in basal medium minus nitrogen source (MIN -N), basal medium plus amino acids plus chloramphenicol (MIN + AA + CHL.), basal medium plus amino acids plus dinitrophenol (MIN + AA + DNP), and basal medium plus amino acids MIN + AA) These *cultures* were incubated at 37°C and samples withdrawn at five-minute intervals of incubation Immediately after with-drawal, these samples were plated on an agar medium allowing expression of both total survivors and mutants The upper curve (MIN. + AA) traces the progress of mutation stabilization The two lower curves (MIN − N. and MIN + AA + CHL.) trace the progress of mutation frequency decline.

RNA chains which unwind from each other and from the replica DNA. These RNA templates are then available for new mating partners, or may be used for heterocatalytic activities. The replica DNA could now act as a template for the synthesis of more RNA protein chains.

In our laboratories we have sought, by studying the intracellular processes involved in establishment of gene mutations in the hereditary structures, to learn something of the biochemical processes involved in genetic replication. These experiments have been carried out on various strains of *E. coli* bacteria. Investigators have found repeatedly that the induced mutation frequency at first increases linearly with the radiation dose, but as these doses are increased to higher levels, the mutation frequency reaches a plateau (for review of the literature see Zelle, 1955). This suggested to us that some intracellular material is modified by radiation prior to the mutational event. The plateauing gives the impression that this material is limited, and that at high doses of radiation the mutation-induction process is running out of substrate. Seizing on this point of attack we sought to establish the nature of the radiation-sensitive material through a series of preirradiation growth factor supplementation experiments. Bacteria, synchronized in growth and cell division, were fed various growth factors immediately before exposing them to ultraviolet. Following irradiation, they were plated in a complete growth medium which allowed expression of the mutation being studied (see Haas and Doudney, 1957, for experimental details).

The results of this series of experiments indicated that the principal factors involved in the mutation process are the purines and pyrimidines. When these substances are furnished to the bacteria prior to radiation exposure, the induced mutation frequency rises with increasing radiation dose much more rapidly, and to a higher final level, than is the case with bacteria grown in their absence (Fig. 3). It was established that the combination of uracil and cytosine plus either adenine or guanine is required for this effect. It was also found that the ribosides of these compounds are more effective than the free bases. Of considerable interest was the finding that thymine or the deoxyribosides are completely ineffective in increasing the induced mutation frequency. Also, ribosides or free bases added subsequent to irradiation usually decreased the expected mutation frequency. There was no correlation between the nucleic acid content of the cell and the mutational response; and cells incubated either in the presence or absence of the purines and pyrimidines exhibited comparable

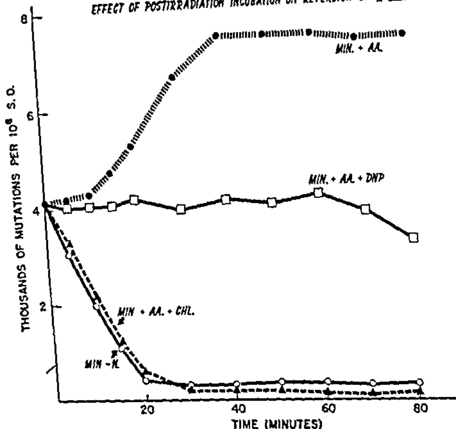


Figure 4 Effect of postirradiation incubation in different media on ultraviolet induction of reverse mutations in *E. coli* strain WP2. Immediately following irradiation equal aliquots of the bacterial culture were suspended in basal medium minus nitrogen source (MIN.–N.), basal medium plus amino acids plus chloramphenicol (MIN. + AA. + CHL.), basal medium plus amino acids plus dinitrophenol (MIN. + AA. + DNP.), and basal medium plus amino acids (MIN. + AA.). These cultures were incubated at 37°C and samples withdrawn at five-minute intervals of incubation. Immediately after withdrawal, these samples were plated on an agar medium allowing expression of both total survivors and mutants. The upper curve (MIN. + AA.) traces the progress of mutation stabilization. The two lower curves (MIN. – N. and MIN. + AA. + CHL.) trace the progress of mutation frequency decline.

RNA chains which unwind from each other and from the replica DNA. These RNA templates are then available for new mating partners, or may be used for heterocatalytic activities. The replica DNA could now act as a template for the synthesis of more RNA protein chains.

In our laboratories we have sought, by studying the intracellular processes involved in establishment of gene mutations in the hereditary structures, to learn something of the biochemical processes involved in genetic replication. These experiments have been carried out on various strains of *E. coli* bacteria. Investigators have found repeatedly that the induced mutation frequency at first increases linearly with the radiation dose, but as these doses are increased to higher levels, the mutation frequency reaches a plateau (for review of the literature see Zelle, 1955). This suggested to us that some intracellular material is modified by radiation prior to the mutational event. The plateauing gives the impression that this material is limited, and that at high doses of radiation the mutation-induction process is running out of substrate. Seizing on this point of attack we sought to establish the nature of the radiation-sensitive material through a series of preirradiation growth factor supplementation experiments. Bacteria, synchronized in growth and cell division, were fed various growth factors immediately before exposing them to ultraviolet. Following irradiation, they were plated in a complete growth medium which allowed expression of the mutation being studied (see Haas and Doudney, 1957, for experimental details).

The results of this series of experiments indicated that the principal factors involved in the mutation process are the purines and pyrimidines. When these substances are furnished to the bacteria prior to radiation exposure, the induced mutation frequency rises with increasing radiation dose much more rapidly, and to a higher final level, than is the case with bacteria grown in their absence (Fig. 3). It was established that the combination of uracil and cytosine plus either adenine or guanine is required for this effect. It was also found that the ribosides of these compounds are more effective than the free bases. Of considerable interest was the finding that thymine or the deoxyribosides are completely ineffective in increasing the induced mutation frequency. Also, ribosides or free bases added subsequent to irradiation usually decreased the expected mutation frequency. There was no correlation between the nucleic acid content of the cell and the mutational response; and cells incubated either in the presence or absence of the purines and pyrimidines exhibited comparable

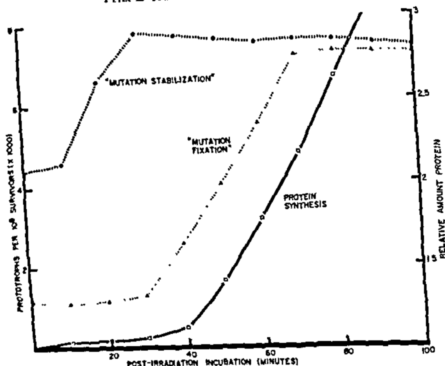


Figure 5. Time relations between the processes of mutation stabilization and mutation fixation in *E. coli* strain WP2. The "Mutation Stabilization" curve was obtained by incubating the ultraviolet-irradiated culture at 37°C in basal medium supplemented with amino acids immediately following irradiation. Aliquots of this culture were withdrawn at ten-minute intervals and plated on agar medium allowing expression of survivors and mutants. The "Mutation Fixation" curve was obtained by withdrawing similar aliquots from the above culture at ten-minute intervals and adding chloramphenicol instead of plating immediately. These cultures were incubated for thirty minutes in chloramphenicol medium and then plated on the same agar medium as above. The "Protein Synthesis" curve was obtained by determining the relative amount of protein in the culture at each ten-minute interval. The mutation employed was reversion to prototroph in tryptophan-requiring *E. coli* WP2.

quency decline (Doudney and Haas, 1959). Figure 5 also shows that potential mutations are stabilized in the cell for a considerable time period prior to occurrence of mutation fixation, and during this period they are subject to conditions promoting mutation frequency decline. After 30 minutes' incubation the numbers of potential mutations subject to mutation frequency decline start to decrease, and after 70 minutes mutation fixation is completed. Neither mutation

nucleic acid content per cell, but widely differing induced mutation frequencies. A number of factors in these experiments have indicated that DNA formed prior to radiation exposure cannot be responsible for the increased mutation frequency, and that preformed nucleic acid probably is not involved at all in ultraviolet-induced mutation.

There is also considerable evidence that mutation is not an instantaneous event occurring immediately on contact of the inducing agent with genetic structures (see Witkin, 1956, for review of the evidence). Therefore, we turned our attention from the preirradiation conditions influencing mutation induction to study of the post-irradiation events involved in the establishment of the mutations in the genome. It was demonstrated that in the absence of adequate nitrogen source, or with chloramphenicol treatment (blockage of protein synthesis) an immediate and rapid decline in ultraviolet-induced mutation frequency takes place. We have designated this process "mutation frequency decline" (Fig. 4). The mutation frequency decline process can be prevented by a complex supply of amino acids; and this suggests that amino acids are involved in intracellular temporary stabilization of the potential mutagen (the altered purine or pyrimidine) prior to its utilization in processes leading to mutation induction. Without intending any implication as to mechanism this process has been designated "mutation stabilization" (Doudney and Haas, 1958, 1959; Haas and Doudney, in press). Oxidative phosphorylation is necessary for both mutation stabilization and mutation frequency decline since dinitrophenol (DNP), which interferes with oxidative phosphorylation, prevents both processes (Haas and Doudney, in press).

These results accumulatively lend support to the idea that mutation response to a given dose of ultraviolet depends on (1) the intracellular supply of nucleic acid precursors during ultraviolet exposure and (2) *following exposure, the relative efficiency of at least two competitive processes for the ultraviolet-altered precursors.* One process is amino acid dependent and leads to stabilization of the mutagenic material; the other is amino acid independent and removes the mutagenic substrate from pathways leading to mutation induction.

Figure 5 demonstrates the postirradiation incubation time relation between mutation stabilization and subsequent mutation fixation in the case of reverse mutation of tryptophan requirement in *E. coli* strain WP2. The mutation is considered fixed if it is no longer subject to the reversing effect of chloramphenicol, nitrogen deprivation, photoreversal, or other agents or conditions promoting mutation fre-

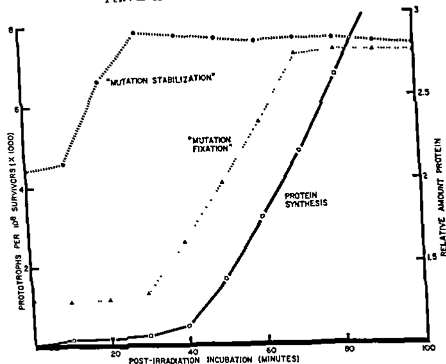


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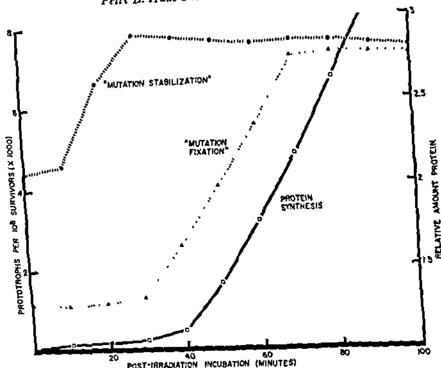


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stabilization nor fixation appears to be correlated with gross protein synthesis.

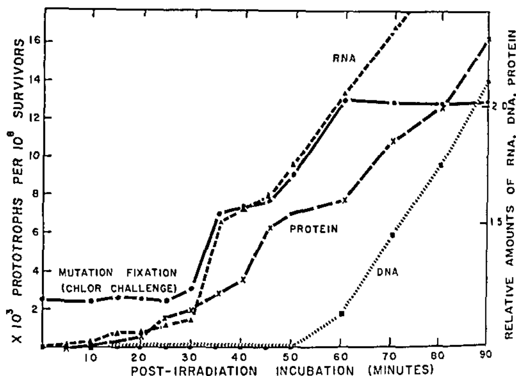


Figure 6 Time relations between mutation fixation, and syntheses of RNA, DNA, and protein in ultraviolet-induced mutation in *E. coli* WP2. Following ultraviolet irradiation the bacteria were incubated at 37°C in basal medium supplemented with amino acids At five-minute intervals samples were withdrawn and subjected to chloramphenicol challenge as in Figure 4 so as to determine the frequency of mutations "fixed" after each period of incubation. At each of the same intervals, samples of the culture were analyzed for RNA, DNA, and protein content. The mutation employed was reversion to prototroph in tryptophan-requiring *E coli* WP2.

We have previously suggested that the processes of mutation fixation may involve RNA synthesis. Experiments were therefore carried out to clarify the relation of mutation fixation to the macromolecular synthetic activities of irradiated bacteria. Figure 6 relates mutation fixation (determined by chloramphenicol challenge) to RNA, DNA, and protein syntheses in an irradiated culture of bacteria The progression of mutation fixation is closely related with RNA synthesis. Fixation is initiated at about the same time that RNA synthesis begins, and attains its maximum level by the time the net amount of RNA has doubled. In this particular experiment the rate of RNA

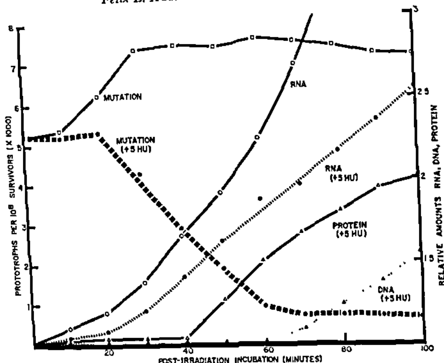


Figure 7 Effect of 5-hydroxyuridine on the postirradiation fixation of ultraviolet-induced reverse mutations to tryptophan-requiring in *E. coli* strain WP2. Following ultraviolet irradiation, identical aliquots of the irradiated bacteria were incubated at 37°C in basal medium supplemented with amino acids (Mutation curve), and in the same medium to which 5γ/ml 5-hydroxyuridine had been added (Mutation + 5HU curve). At ten-minute intervals of incubation aliquots were withdrawn from each culture and plated to determine the surviving bacteria and number of prototrophic mutants At each time, aliquots were also analyzed for RNA, DNA, and protein syntheses

synthesis shows a decrease during the 35-to-45-minute period of incubation, and a corresponding change in the course of mutation fixation simultaneously occurs. It is quite interesting that this change in RNA synthetic rate at 35 to 45 minutes of incubation is reflected in a corresponding change in protein synthesis rate at the 45-to-60-minute incubation period. This probably means a direct sequential relation of RNA synthesis to protein synthesis. It is important to note that DNA synthesis does not resume until after the processes leading to mutation fixation have been completed.

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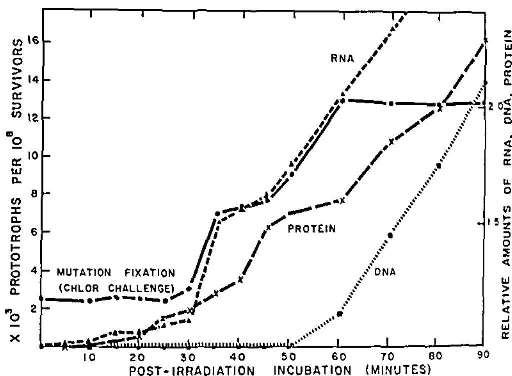


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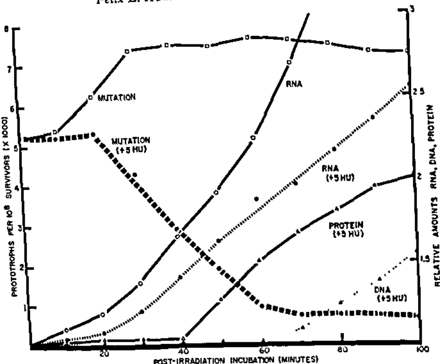


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plied during postirradiation incubation. The effects of this analogue on mutation frequency are shown in Figure 7. The 5-HU-induced decline does not start immediately, but only after 20 minutes' incubation, and it is correlated in a reverse manner with the mutation fixation process. This type of decline involves a 20-minute lag, and the ensuing mutation frequency decline is not completed until after 60 minutes of incubation. Initiation of this decline process always takes place with initiation of RNA synthesis, and is completed at about the time that the relative amount of RNA has doubled. It appears that neither protein synthesis nor DNA synthesis is directly involved. And it seems likely that 5-hydroxyuridine is incorporated during RNA synthesis to produce "nonfunctional" RNA, thus using up the mutagenic substrate.

These experiments suggest that the initial mutagenic event following ultraviolet alteration of nucleic acid precursors involves RNA synthesized from these precursors. It seems reasonable from these studies that RNA synthesis is intermediate to DNA synthesis if we assume that genetic information must ultimately reside in the DNA. It also appears to us that RNA and possibly protein may mediate transfer of information from parent to daughter genetic DNA. While in general our data tend to support the Stent hypothesis for genetic replication (Stent, 1958), there is one step in his theory which appears to us rather unlikely, at least for higher organisms, and probably also for bacteria. That is the formation of the RNA identical duplex.

The improbability for such a structure resides in the numerous elements of chance involved in its formation and action. In the first place, a given RNA strand is liberated at random into nuclear or cytoplasmic milieu. This must then be followed by a successful search for another strand of exactly the same base sequence so as to form the identical duplex. Following the formation of the identical RNA duplex, the structure, or else the DNA formed on it, must find its way to a very specific location during assembly of the total new genetic structure. It does not seem reasonable that the accuracy observed in genetic replication would be possible if such random processes were involved. If this were the case, there would probably be so many mistakes and failures that exact replication of the genetic structure would be the exception rather than the rule. It seems much more likely that all intermediate steps and products in DNA replication are very closely associated and more or less "anchored" to one spatial site until the completion of replication.

Genetic Replication and Cancer Etiology

There is considerable evidence that approximately the same processes are involved in induction of experimental animal tumors as those shown to be required for establishment of induced mutations in bacteria. Rogers has investigated the mechanisms of ultraviolet, nitrogen mustard, urethan, and methylcholanthrene induction of mouse cancer (Rogers, 1955a, 1955b, 1957a, 1957b, 1959). He has established that these tumor-inducing agents act through nucleic acid synthesis, and that their carcinogenic effectiveness depends on the mitotic stage of cell division. Neoplastic change could be initiated only in cells actually in the process of nuclear division, or in interphase when DNA is being synthesized (Rogers, 1959). Rogers has advanced a hypothesis for induction of mouse pulmonary adenomas almost identical with those which we have advanced for mutation induction in bacteria In his urethan experiments it was found that a single large injection of DNA hydrolysate, given immediately prior to urethan exposure, greatly reduced the incidence of urethan-induced tumors—presumably by diluting out intracellular carcinogens produced by urethan from DNA precursors. Also, aminopterin (known to inhibit nucleic acid synthesis), given at the time of urethan injection, greatly increased the incidence of urethan-induced tumors; but this increase also could be mitigated by injection of nucleic acid hydrolysates The most effective antagonists of urethan action were found to be the pyrimidines or their precursors. Rogers believes that the carcinogen is an atypical pyrimidine produced by the action of urethan on DNA precursors, which is either incorporated into the nucleic acid and henceforth resynthesized and passed to daughter cells, or, alternatively, exerts a temporary interference during the synthesis of new DNA, thus changing the nucleotide sequence (Rogers, 1957b)

Studies on a number of radiomimetic agents have led most investigators to conclude that the mutagenic and carcinogenic actions of these chemicals are one and the same, and that they exert their effects during the synthesis of new DNA (see Haddow, 1953, for review of the evidence). In regard to the hormonal induction of cancer, it is of significance that most hormones considered carcinogenic are the growth-stimulating hormones (Gardner, 1953). It may be that these act simply by speeding up cellular proliferation, thus increasing the chances of mutation per unit time. These same hormones also produce extensive tissue degradation, however; therefore,

they also may act to supply the rapidly proliferating cells with abnormal nucleic acid precursors at a time when the genetic replication is greatest. Abnormal nucleotides or pyrimidines produced by hormonal action may be just as effective mutagens as those produced by radiation or chemical action. The organ specificity of hormone-induced tumors is probably due only to the fact that these tissues have more contact with the hormone or its metabolic derivatives. The same is true for most other tumor-inducing agents: Urethan induces mainly lung tumors; Bittner virus, mainly mammary tumors; Rous virus, connective tissue tumors; and butter yellow, liver tumors.

As regards the tumor viruses, despite implication as initiating agents for certain tumors, their inducing mechanism has remained obscure. Two factors of importance to this discussion have become apparent, however. First, the virus is necessary *only for tumor induction.* Once the initiating change has been induced in a cell, the descending cells are tumorous, and the virus is no longer necessary. Second, many other carcinogenic agents can induce tumors *indistinguishable* from those which are virus-induced, *and from which no virus can be isolated.* Both of these facts indicate that viruses are probably only one of a number of agents which attack the same cellular mechanism controlling growth and differentiation. In this aspect viral induction of cancer becomes the equivalent of mutation. The bacteriophage-transduction and the lysogenic-bacteria data have shown that very often close and intimate associations between the genetic structures of viruses and host cells can be expected. These data have indicated mechanisms by which virus genetic material or foreign-host genetic material may actually be incorporated into the genome of the host cell. Therefore, it is not reasonable to consider the viral theory and the somatic mutation theory of cancer induction as mutually exclusive. Explanations must be sought which will allow for both theories, and all the indications are that this common ground will be found in the processes of genetic replication.

The evidence is ever increasing that the initial step in carcinogenesis, regardless of inducing agent, involves DNA and a permanent alteration of the genetic mechanism. It is quite probable that the major part of these carcinogenic changes take place during the *synthesis* of new genetic DNA. If this proves to be so, it is difficult to see just how cancer can be prevented, since any preventive measure that can be visualized would also halt essential cellular processes in normal cells as well; and we will necessarily have to concentrate our efforts on eradication or control of tumorous growth after it has ap-

peared. However, there is much to be gained in learning the processes involved in genetic replication, and in determining what errors in these processes can result in neoplastic growth. The types of information obtained in such studies will certainly be a necessary prerequisite for any proposed scheme for the eradication or control of cancer.

ACKNOWLEDGMENTS

This research was supported in part by Research Grant C-3323 from the National Cancer Institute division of the U S. Public Health Service and by U. S Atomic Energy Commission Contract AT-(40-1)-2139

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Relation of Chromosome Status to the Origin and Progression of Tumors: The Evidence of Chromosome Numbers

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Modern methods for chromosome analysis started coming into use in cancer research in 1950. Since then, chromosome analyses have been made for a number of normal and malignant cell populations, making a survey of the results profitable at the present moment. Ascites tumors were the first materials to give favorable chromosome pictures, and they still are the material best understood. Recently, interest has focused on normal and malignant cells in culture: As primary explants they may give information about the chromosome status of cells in the body; as long-term cultures they have a bearing on problems of cancerogenesis and tumor progression.

Normal tissues *in situ* have long been considered a difficult material for chromosome study. Opinions diverge, even in recent years, about basic properties, such as the detailed composition of the idiograms, the stability of the karyotype during somatic differentiation, etc The chromosomes of solid tumors and precancerous stages are still almost unknown, although newly worked-out methods give good promise for these materials (Ford and Hamerton, 1956; Bayreuther and E Klein, 1958, Tjio and Puck, 1958a).

In the present paper I will deal mainly with chromosome numbers. They constitute a property easily observable and analyzable. It is true that chromosome numbers by themselves are of limited interest. They may give valuable information, however, of genetic stability, or variability, of a tissue. These are properties that are significant for the

interpretation of evolutionary trends in a cell population, trends that are at the basis of any selective adaptation of a tissue. Besides, a variability in chromosome number will hardly be of any lasting consequence, either in populations of cells or in populations of organisms, unless associated with structural and genic variation.

Without claim of completeness, I have brought together data from the last decade on chromosome numbers in normal cells and cancer cells. These data, comprising some 300 cases of chromosome number distributions, are presented as eleven tables. The tables contain, maximally, the following columns:

1. Case Number
2. Tumor (Tables 6, 7); Cell strain (8, 9)
3. Reference to literature
4. Species (1, 2)
5. Tissue (1, 2); Tissue of origin (8, 9); Tumor line (3–7); Tumor type (11); Passage Number (10)
6. Variation extremes for chromosome numbers
7. No-cell range, separating the *s* and 2*s* regions (3, 6)
8. Different numbers (number of different counts)
9. *s* (the stemline number)
10. Percent of cells with *s*
11. Number of metacentrics (8)
12. Number of cells underlying the chromosome counts

The columns of greatest significance in characterizing each case are columns 6 and 9, indicating extent of variation and level of stemline, respectively. Columns 8 and 10 are estimates of variability: The lower the value of 8, and the higher the value of 10, the more constant the chromosome number. Column 12 is important for judging the safety of the other values. Especially in Table 9 (human cell strains) many cases were included based on few or single counts. This was done because it was thought that even solitary, safe chromosome counts were of piloting interest in these cases.

1. Normal Cells

Chromosome counts of normal cells are presented in Table 1. In 1951 Therman and Timonen made their claim of a wide chromosome number variation in somatic tissue of man, especially in endometrium (Table 1, Case 1). Again, a similar, although less extreme, variation was observed for this tissue in 1954 by Manna (Table 1, Case 12). Tanaka (1953) had corresponding experiences for rat tissues: In

regenerating liver, for instance, 52 different chromosome numbers were recorded (Table 1, Case 3).

Contrary to this, Sachs in 1954 (Table 1, Cases 13–16), Hungerford (1955, Cases 21–22), Beatty (1957, Cases 25–26), and others, found somatic tissues to be remarkably constant in chromosome number. Their results indicated that the wide chromosome number variation reported for somatic tissues might have been due to unsatisfactory technique, that is, mainly losses of chromosomes at squashing. With the kind permission of Dr. Tjio I have included in Table 1 his unpublished data on 13 tissues of man, rat, mouse, and American opossum (Cases 30–42). Altogether, in some 2,000 exact counts Tjio found, except for some polyploid numbers, no single number deviating from the expected somatic number of the species, thus a very high degree of stability.

Although I attach great significance to Tjio's results, part of which I have had the privilege of following at our laboratory in Lund, I do not interpret them as implying that no variations by *single chromosomes* can occur. I am convinced that many of the deviations from the normal number, reported by various authors, are real. To mention one instance, the trisomic numbers in mouse cells counted by Hungerford (Table 1, Cases 21, 22) are unquestionable. Small deviations undoubtedly occur; nevertheless, the chromosomal and mitotic stability of normal tissues is evidently high. Ford, Hamerton, and Mole (1958) express a similar opinion, based on their observations on mouse bone marrow, spleen, and lymphoid tissue (Table 1, Cases 27–29). Having counted several numbers below 2x but very few above this number, they interpret this skewness as due to chromosome losses at squashing. Their conclusion is: "The rare occurrence of elimination and nondisjunction is not excluded, of course, but the considerations just given form the basis for our belief that, in normal cells of the four tissues examined, true deviation from the standard diploid number of 40 is exceptional, if it occurs at all" (*loc. cit.*, p. 242)

In summary: Chromosome conditions are highly stable in normal tissues Deviating numbers occasionally formed are of no selective value under normal conditions, and they will therefore disappear from the tissue, overgrown by cells with normal karyotype. In other words, the stemline number for normal tissues in the body is $= 2x$.

In justice to authors claiming a high degree of chromosome number variation, the possibility must not be overlooked that chromo-

TABLE 1. Chromosome Numbers of Various Normal Tissues (Cases 1 to 42)

1 No	3. Reference	4. Species	5. Tissue	6. Variation Extremes	8. Different Numbers	9. s	10. % Cells with s	12. Number of Cells
1	Therman & Timonen, 1951	Man	Endometrium	4-104	-	20-25	28	1000
2	Tanaka, 1953	Rat	Liver	37-84	12	42	67	149
3			Regenerating liver	36-89	52	42	31	1163
4			Spleen	39-48	7	42	83	60
5			Brain	42-47	3	42	96	52
6			Lung	42-43	2	42	97	31
7			Heart	36-46	3	42	94	32
8			Amnion	42	1	42	100	20
9			Bone marrow	36-49	12	42	68	73
10			Kidney	39-46	6	42	82	61
11			Testis	42	1	42	100	30
12	Manna, 1954	Man	Endometrium	27-68	19	48	28	100
13	Sachs, 1954	Man	Endometrium	48	1	48	100	50
14		Field mouse	"	50	1	50	100	50
15		Rat	"	42	1	42	100	50
16			Cornea	42	1	42	100	20
17	Walker & Boothroyd, 1954	Man	Endometrium	42-62	17	50	13	40
18		Mouse	Intestine, young	30-48	16	39	18	63
19			" adult	36-45	10	39	20	40
20			Cornea	[illegible]	[illegible]	[illegible]	25	40

21	*Hungerford, 1955*	*Mouse*	*Embryonic cells*	40–41 x)	2	40	98	99
22			Testis	40–41	2	40	99	100
23	Tonomura & Yerganian, 1956	Chinese hamster	Regenerating liver	18–25 x)	7	22	50	98
24	1957		Bone marrow	20–24	5	22	68	102
25	Beatty, 1957	Mouse	Cornea, exact	40	1	40	100	20
26			approxim	37–46	9	40	42	445
27	Ford, Hamerton, & *Mole*, 1958	*Mouse*	Bone marrow	<30–41	>12	40	84	646
28			*Spleen*	<30–41	>11	40	72	207
29			*Lymphoid*	34–40	6	40	78	54
30	Tjio, unpublished	Man	Liver	46 x)	1	46	100	45
31			Testis	46	1	46	100	54
32		Mouse	Liver	40 x)	1	40	100	244
33			Regenerating liver	40 x)	1	40	100	326
34			Spleen	40 x)	1	40	100	250
35			Brain	40	1	40	100	56
36			Lung	40	1	40	100	120
37		Rat	Liver	42 x)	1	42	100	154
38			Regenerating liver	42 x)	1	42	100	107
39			Spleen	42 x)	1	42	100	96
40			Brain	42	1	42	100	42
41			Lung	42	1	42	100	111
42		American opposum	Testis	22	1	22	100	210

x) *In addition, polyploid numbers*

TABLE 1. Chromosome Numbers of Various Normal Tissues (Cases 1 to 42)

1 No	3. Reference	4. Species	5. Tissue	6. Variation Extremes	8. Different Numbers	9. s	10. % Cells with s	12. Number of Cells
1	Therman & Timonen, 1951	Man	Endometrium	4-104	-	20-25	28	1000
2	Tanaka, 1953	Rat	Liver	37-84	12	42	67	149
3			*Regenerating liver*	*36-89*	52	42	*31*	*1163*
4			Spleen	39-48	7	42	83	60
5			Brain	42-47	3	42	96	52
6			Lung	42-43	2	42	97	31
7			Heart	36-46	3	42	94	32
8			Amnion	42	1	42	100	20
9			Bone marrow	36-49	12	42	68	73
10			*Kidney*	*39-46*	6	*42*	82	61
11			Testis	42	1	42	100	30
12	Manna, 1954	Man	Endometrium	27-68	19	48	28	100
13	*Sachs, 1954*	Man	Endometrium	48	1	48	100	50
14		Field mouse	"	50	1	50	100	50
15		Rat	"	42	1	42	100	50
16			Cornea	42	1	42	100	20
17	Walker & Boothroyd, 1954	Man	Endometrium	42-62	17	50	13	40
18		Mouse	Intestine, young	30-48	16	39	18	63
19			" adult	36-45	*10*	39	*20*	40
20			Cornea	36-45	8	40	25	40

TABLE 2 *Primary Cultures and Early Passages* (Cases 43 to 68)

1 No.	3 Reference	4 Species	5 Tissue	6 Variation Extremes	8 Different Numbers	9 s	10 % Cells with s	12 Number of Cells
43	Hsu, 1952	Man	Spleen	44–49, 91	7	48	73	124
44	Tjio & Levan 1956a	Man	Lung	46	1	46	100	261
45	Hsu, Pomerat, & Moorhead, 1957	Man	*Synovial* lining of the leg	44–47, 92	5	46	69	45
46	Syverton 1957	Man	Lung, liver, adrenal	45–46	2	46		11
47			Palate fibrocyte	46	1	46	100	20
48	Ford, Jacobs, & Lajtha, 1958	Man	Bone marrow	42–50	9	46	66	555
49	*Levan & Biesele, 1958*	Mouse	Spleen	40–41, 75–82, 160	6	40	66	77
50			Liver	40–42, 80	4	40	57	21
51			Brain	40–42, 75–82, 115, 160	9	40	28	115
52			Skin	39–42, 73–83, 160	15	40	55	150
53	Tjio & Puck, 1958a	Chinese Hamster	Spleen	21–23, 44	4	22	90	568
54			*Ovary*	21–23, 44, >44	6	22	84	531
55		*Opossum*	Testis	22, 44	2	22	99	212
56		Man	Cervix	46, 92	2	46	88	274
57			Skin, prepuce, uterus	46, 92	2	46	90–96	-
58	*Tjio & Puck, 1958b*	*Man*	*Skin*	45–46	2	46	99	689
59			Lung	46	1	46	100	75
60			*Prepuce*	46	1	46	100	112
61			Testis	46	1	46	100	105
62			*Cervix*	46–47	2	46	99	486
63			*Myo- & Endometrium*	46	1	46	100	138
64			*Ovary*	46	1	46	100	112
65	Levan, unpubl	*Man*	Minced embryo, mostly fibroblast	45–47, 91	4	46	70	25
66			Skin, adult fibroblast	46, 92	2	46	60	5
67			Amnion	44–48, 65–75, 92	7	46	77	43
68			Kidney	46	1	46	100	5

some conditions may be less stable in tissues with an ephemeral life span, such as endometrium, where no necessity exists for upholding genetic continuity. Recently, two German writers (Weicker and Terway, 1958) found it probable that a gradual decrease in chromosome number takes place during erythropoiesis of the Chinese hamster, so that the only strictly diploid cells are the proerythroblasts, all later stages being hypodiploid. Their pictorial documentation is good, although not yet conclusive.

Normal tissues, explanted *in vitro,* should at least in the beginning give a true representation of the normal somatic chromosomes of the animal species in question. Kemp, in 1929, analyzed the human somatic chromosomes in primary explants of embryonic spleen, liver, and heart. Although newly explanted tissues are less favorable for chromosome study than old well-adapted cell strains, recent technical advances have made them accessible. Thus, Hsu in 1952 introduced the very significant methodologic improvement of hypotonic pretreatment, thereby initiating the modern chromosome study of tissue cultures.

As seen from Table 2, in which data on chromosome numbers of primary cultures and early passages are collected, the chromosome variation is somewhat more pronounced than in the body. Furthermore, the numeric variation is associated with incidental structural changes. The stemline number usually remains at 2*x* in primary explants, but numbers above 2*x*, around and especially below 4*x*, are frequent in some cases. Levan and Biesele (1958) observed in embryonic mouse cell cultures that the tetraploid fraction of the tissues was occasionally favored by the explantation (Table 2, Cases 49–52).

In early passages of human amnion, grown in the laboratory of Miss Leila Diamond at the Sloan-Kettering Institute, cells with the normal number sometimes contained structurally new chromosomes, and deviating numbers were observed. In one culture tube many mitoses had numbers around 70 (Table 2, Case 67), indicating that the nucleus of a new cell strain was under development. Cases like this indicate the hazards of making karyotype descriptions from single cells of an explant. The first mitosis after explantation may contain chromosomes with altered structure.

Is it possible to maintain the normal genotype, and karyotype, in tissue culture? Tjio and Puck (1958a), discussing this point, are of the opinion that their cultures of man and of the opossum remained unchanged for more than five months. However, their Chinese ham-

TABLE 3. Hyperdiploid Ehrlich (Cases 69 to 94)

o	3 Reference	5 Tumor Line	6 Variation Extremes	7 No-Cell Range	8 Different Numbers	9 s	10 % Cells with s	12 Number of Cells
3	Bayreuther, 1952	E.L.	41-96	50-89	11	45-46	54	100
0	Kaziwara, 1954	E L.	43-94	49-89	11	46	32	100
1		E L 88	44-100	49-81	21	91	13	100
2	Tjio & Levan, 1954	Landschutz I	38-98	65-77	20	46	74	182
73		Landschütz II	33-50	-	8	46	81	108
74		E L	41-90	65-89	13	45	33	100
75	Querner, 1955	E L	17-85	49-72	18	43	14	49
76		Clone V	28-94, 145	50-65	26	43	15	100
77		VI	23-83	48-75	23	40	18	100
78		VII	37-93	50-69	16	44	24	100
79		VIII	34-92	61-66	23	43	16	150
80		IX	31-89	59-65	25	43	15	100
81	Feldman & Sachs, 1958	"6C3HED-ICR"	44-90	47-89	4	45	72	50
82	Hansen-Melander, 1958	Landschutz Clone a	40-96	66-81	22	46	47	99
83		b	40-102	66-71	18	46	38	100
84		c	42-94	56-87	15	46	55	100
85		d	43-96	57-83	11	46	66	85
86		21/119	44-100	66-70	19	47	49	100
87		30/172	43-90	55-87	10	45	76	99
88		Lz + 21/119	42-94	55-82	12	47	38	100
89		Lz + 30/172	42-96	57-95	10	45	38	100
90		21/119 + 30/172	42-94	71-89	15	47	33	100
91		Lz + 21/119 + 30/172	41-100	49-85	10	47	41	100
92	Ising, 1958	Landschutz	44-51	-	7	46	73	200
93		In hamster	37-51	-	10	46	57	319
94		Back in mouse	43-48	-	6	45	39	150

The other is the near-tetraploid Ehrlich, used by Klein in experiments with solid tumor into ascites conversion Krebs-2 is another of Klein's tumors having strikingly similar chromosome properties to the tetraploid Ehrlich

All sublines of Ehrlich Lettré have their stemline close to 45–46 (Table 3). It was used for polyploidization experiments by Kaziwara (1954), who initiated the hypertetraploid subline EL88 by changing the environment for the tumor, thus selectively favoring the 2s

ster cultures started varying at once, after explantation. In a later paper, the same authors (Tjio and Puck, 1958b) claim that various cultures of human tissue showed no variation in chromosome number or morphology for as long as eight months, except for a low frequency of tetraploid cells (Table 2, Cases 58–64). Only two cells out of 1,717 deviated from the number 46, these two counts being due "either to mitotic nondisjunction or to technical accidents" (*loc. cit.*, p. 1231).

Thus, early tissue cultures differ considerably in their degree of chromosome stability. Studying factors involved in the chromosome number variation of tissue cultures is an urgent task. It seems doubtful whether it will ever be possible to duplicate *in vitro* the normal body environment to such a degree as to make normal cells grow and multiply without first undergoing adaptational adjustments. The possibility of eliminating or controlling genic variation *in vitro* of normal mammalian cells would increase immensely the usefulness of them for cytogenetic analysis.

2. Ascites Tumors

Ascites tumors represent far advanced stages of cancer development. They usually are old, transplantable tumors, maintained for years in laboratories. Since they agree in their essential properties with serially grown solid tumors, and with primary ascites tumors in man, they may be taken as representatives of well-adapted cancers in general. Their chromosome conditions have a bearing on important evolutionary trends in neoplasms.

General characteristics of ascites tumors, distinguishing them from normal cells, are evident from Tables 3 through 7. Usually, a wide chromosome number variation is present in all of them, one part of the variation being at the stemline number, the *s* region, another part at the double stemline number, the 2*s* region (see especially Tables 3 and 6). The location of the stemline number generally is heteroploid, that is, it rarely coincides with the somatic number of the species of origin, and if it does, it very probably is cryptoheteroploid. The continuous presence in the tumor population of many genotypes besides the predominant one is at the basis of the remarkable adaptability to the environment of the tumors.

Among mouse ascites tumors, two groups have been extensively used for chromosome studies, both referred to as Ehrlich. One is the hyperdiploid Ehrlich, often called Ehrlich Lettré, or EL, since many of its present sublines go back to Lettré's laboratory at Heidelberg.

TABLE 3. Hyperdiploid Ehrlich (Cases 69 to 94)

	3 Reference	5 Tumor Line	6 Variation Extremes	7. No-Cell Range	8 Different Numbers	9 s	10 % Cells with s	12 Number of Cells
	Bayreuther, 1952	E L	43-96	50-89	11	45-46	34	100
	Kaziwara, 1954	E L	43-94	49-88	11	46	32	100
		E L 88	44-100	49-81	21	91	13	100
	Tjio & Levan, 1954	Landschütz I	38-98	65-77	20	46	74	162
		Landschütz II	23-50	-	8	46	81	108
		E L	41-90	65-89	13	45	33	100
	Querner, 1955	E L	17-85	48-72	18	43	14	49
		Clone V	28-94, 145	50-65	26	43	13	100
		VI	23-83	48-75	23	40	18	100
		VII	37-93	50-69	16	44	24	100
		VIII	34-92	61-66	23	43	16	150
		IX	31-89	59-65	25	43	15	100
	Feldman & Sachs, 1958	"6C3HED-ICR"	44-90	47-89	4	45	72	50
2	Hansen-Melander, 1958	Landschütz Clone a	40-96	66-81	22	46	47	98
3		b	40-102	66-71	18	46	38	100
4		c	42-94	56-87	15	46	55	100
5		d	43-96	57-83	11	46	66	85
86		21/119	44-100	66-70	19	47	49	100
87		30/172	43-90	55-87	10	45	76	99
88		Lz + 21/119	42-94	55-82	12	47	38	100
89		Lz + 30/172	42-96	52-95	10	45	38	100
90		21/119 + 30/172	42-94	71-89	15	47	33	100
91		Lz + 21/119 + 30/172	43-100	49-85	10	47	41	100
92	Ising, 1958	Landschütz	44-51	-	7	46	73	200
93		In hamster	37-51	-	10	46	57	319
94		Back in mouse	43-48	-	6	45	39	150

The other is the near-tetraploid Ehrlich, used by Klein in experiments with solid tumor into ascites conversion. Krebs-2 is another of Klein's tumors having strikingly similar chromosome properties to the tetraploid Ehrlich.

All sublines of Ehrlich Lettré have their stemline close to 45–46 (Table 3). It was used for polyploidization experiments by Kaziwara (1954), who initiated the hypertetraploid subline EL88 by changing the environment for the tumor, thus selectively favoring the 2s

TABLE 4. Near-tetraploid Ehrlich (Cases 95 to 134)

1. No	3. Reference	5. Tumor Line	6. Variation Extremes	8. Different Numbers	9. s	10. % Cells with s	12. Number of Cells
95	Levan & Hauschka, 1952	Philadelphia, early generation	40, 64, 72–91, 100, 143	20	80	18	50
96	Hauschka & Levan, 1958	Gen. 27	75–91	15	83	27	100
97		118	71–90	17	78	20	100
98		138	70–81	12	77	16	50
99		190	68–83	14	76	22	100
100		Stockholm Gen. 282	70–87	17	80	15	80
101	Hauschka, 1958	Ehrlich-S	72–85	13	77	22	100
102		-NMF-R	69–79	10	72	41	100
103	Yosida, 1954a	Osaka University	51–94, 132	17	-	-	24
104	Isirg, 1955	Ea 1, *in mouse*	54–95	24	80	25	698
105		in hamster (234–331 days)	54–83	16	76	39	396
106		back into mouse (30–146 days)	69–85	15	76	51	325
107	Isirg, 1958	Ea 2, in mouse	63–99	21	79	26	320
108		in hamster pass 2–30	70–86	16	78	27	349
109		in cortisone hamster	61–92	22	79	23	398
110		.	67–83	16	78	27	424
111		.	73–86	12	79	28	280
112		Ea 3, in mouse	70–94	14	79	37	250

113		in hamster pass 1–7	67–87	13	78	32	100
114		18–61	69–81	12	77	16	114
115		62–92	67–79	11	74	32	125
116		96–119	64–76	10	72	29	104
117		as contamination	67–85	13	73	29	100
118		in cortisone hamster	74–95	10	79	31	95
119		"	69–84	16	78	20	322
120		in rat pass 11–42	68–84	17	79	21	390
121	Hauschka & Levan, 1958	Clone El Gen. 1	74–95	15	80	20	50
122		5	80–108	13	85	23	100
123		69	80–113	16	84–85	16	100
124		236	77–90	11	82	21	100
125		E2 1	69–96	12	75	28	40
126		5	70–82	11	73	21	100
127		80	69–80	12	73	21	100
128		283	69–75	7	72	47	100
129		E3 5	70–91	13	76	27	100
130		E4 5	74–82	9	76	41	100
131		E5 3	74–81	8	78	27	100
132		E6 3	71–85	12	76	31	100
133		E7 3	71–83	12	75	27	100
134		E8 3	69–83	12	74	22	100

fraction of the population (Cases 70, 71). This experiment is a model for one type of evolutionary change occurring spontaneously in tumors through their adaptational processes.

The EL tumor has provided material for clone experiments by Querner (1955) and Hansen-Melander (1958). The low stemline numbers (40–44) reported by Querner, together with the wide distribution of chromosome numbers, seem somewhat doubtful (Table 3, Cases 75–80). In all other experiments the EL has proved remarkably stable; neither Hansen-Melander in clone experiments (Cases 82–91), nor Ising by heterologous transplantation (Cases 92–94), was able to bring about stemline shifts by more than one chromosome.

The tetraploid Ehrlich family (Table 4) has given interesting information about evolutionary mechanisms. Two sets of materials will be discussed: that of Hauschka used in clone experiments (Cases 95–100, 121–134), and that of Ising used in heterologous transplantation work (Cases 104–120).

Early generations of the Ehrlich stock tumor had the stemline 80, both when carried in America and in Europe (Cases 95, 96). In Sweden this condition is still prevailing (Cases 100, 104, 107, 112), but in Hauschka's laboratory in Philadelphia and in Buffalo the stemline number went down gradually from 80 to 76 (Cases 97–99). This is further emphasized by the fact that all but one of the eight Ehrlich clones had stemlines at hypotetraploid levels (Cases 125–134). Since the tumor has been constantly carried in Swiss/Ha ICR mice in Hauschka's laboratory, while in Sweden it has been grown in varying genotypes, the shift in stemline number in America may be regarded as an adaptation to the Swiss/Ha host genotype.

It is suggestive to compare these chromosomal events with the stemline shifts produced by Ising in the same tumor by heterologous transfers, thus effecting an environmental change similar in kind, but more extreme. In altogether six experimental series she found that *long-term serial growth of the Ehrlich tumor in hamster had the same effect*: viz., a decrease in stemline number (Cases 105, 108, 113–117). In one especially complete and revealing experiment, in which the stemline number gradually fell from 79 to 72 (Cases 113–117), Ising showed by actual chromosome measurements that up to 20 per cent of the total chromosome length per cell was lost during the adaptation. Furthermore, she demonstrated that the decrease in chromosome number was smaller or nonexistent, if the transfer was made through cortisone-treated hamsters (Cases 109–111, 118–119), indicating that antigenic factors were active at the adaptation.

The tetraploid Krebs-2 tumor, out of which Hauschka isolated 13 sublines and clones, exhibits a behavior similar to the Ehrlich (Table 5). The stock tumor stemline goes down from 80 to 74 during its

TABLE 5 Krebs-2 (Cases 135 to 157)

o	3 Reference	5. Tumor Line	6 Variation Extremes	8 Different Numbers	9 s	10 % Cells with s	12. Number of Cells
15	Levan & Hauschka, 1952	Philadelphia, Early Gen	34-171	46	80	9	100
36	Hauschka & Levan, 1958	Gen 2	54-84	15	81	18	50
37		50	64-86	15	82	16	50
38		123	70-83	11	74	28	50
39		207	70-78	9	74	28	50
40	Hauschka & Levan, 1958	Clone A Gen 1	62-98	14	75	27	45
41		B 1	55-116	16	75	22	94
42		C 1	62-80	14	73	18	76
43		3	67-74	8	72	20	50
144		107	69-76	8	72	22	50
145		D 1	70-86	13	75	22	51
146		5	70-78	8	73	42	50
147		101	60-88	11	72	26	50
148		E 4	70-76	7	74	34	50
149		F 4	68-81	12	75	28	50
150		G 4	69-77	9	73	32	50
151		H 1	70-90	10	74	21	42
152		4	69-78	10	74	24	50
153		I 4	70-89	11	74,75	26	50
154		K 3	63-81	12	71,72	26	50
155		L 2	67-83	11	71	34	50
156		M 3	66-78	12	73	30	50
157		N 4	72-78	7	75	34	50

adaptation to the host genotype (Cases 135–139). The clones were all in the hypotetraploid region 71 to 75 (Cases 140–157). The stem-line shifts observed may be summarized as follows (including Cases

101–102, which involve the adaptation of an Ehrlich line to the chemotherapeutic agent N-methylformamide):

Tumor line		*Original stemline*	*Stemline after adaptation*
Ehrlich stock		80	76
Ehrlich-S, Ehrlich NMF-R		77	72
Ehrlich clone	1	85	82
	2	75	72
Ehrlich heterologous transplantation			
	Series 1	80	76
	2	79	77
	3	79	76
	4	79	78
	5	79	78
	6	79	72
Krebs-2 stock		80	74
Krebs-2 clone C		73	72
D		75	72

Evidently, in these two tumors, the adaptation to new environments takes place by decrease in chromosome number, and, as shown by Ising, by actual loss of chromosome matter. Ising's observations would be compatible with the occurrence of many small random losses of chromosome segments, most of them acentric, but of some centric ones, too. This suggests that in this case the evolutionary mechanism is the discarding of deleterious genes, rather than the building up of favorable genes. It stands to reason that the elimination of inhibitors should be a simpler process than the new formation of stimulators.

The frequent presence of hypotetraploid numbers in old well-adapted tumors, as well as results from the adaptation *in vitro* of normal mouse cells to cancer (see Table 10), may indicate that this evolutionary trend is of general significance in the adaptation of the mouse genotype. A tetraploid, or near-tetraploid, starting point would facilitate this method of evolution because of the greater capacity of tetraploid cells for compensating for genetic losses.

Some mouse ascites tumors not included in the preceding tables are collected in Table 6, most of them being near-diploid, some being near-tetraploid. The table includes some cases in which the stemline coincides with the somatic number of the mouse (Cases 158, 159,

161–165, 167, 169) or the double somatic number (Cases 160, 184). In some of the near-diploid tumors, sublines occur in the tetraploid region, either artificially produced (Case 160) or spontaneous (Cases 171, 172).

TABLE 6 Mouse Ascites Tumors (Cases 158 to 184)

1 No	2 Tumor	3 Reference	5 Tumor Line	6 Variation Extremes	7 No-Cell Range	8 Different Numbers	9 s	10 % Cells with s	12 Number of Cells
158	6C3HED	Levan & Hauschka, 1953	in C3H/Sc	36–86, 130	46–77	15	40	32	50
159		Hauschka et al., 1956	in ICR Swiss	37–80	47–71	11	40	27	26
160			in DBA/2	40–91	51–70	17	80	14	28
161		Feldman & Sachs, 1958	in C3H/Sc	38–82	46–79	8	40	70	50
162	My Carc	Yosida, 1954b		33–54	-	14	40	25	32
163	Plasma cell leukemia 70429	Hauschka 1958	S	39–42	-	4	40	80	100
164			AM-R	39–41	-	3	40	89	50
165			AM-D	39–42	-	4	40	85	100
166	Mast cell	Hauschka 1958	S	39–43	-	5	41	44	100
167	Turner P815		DON R I	39–42	-	4	40	71	100
168			II	39–44	-	6	41	43	100
169	TA3	Levan & Hauschka, 1953	Philadelphia	38–85	43–53, 55–74	10	40	42	128
170		Levan, 1956a	Ha	40–43	-	4	41	79	100
171			Kl	64–74	-	10	68	36	200
172			Ko	66–75	-	10	70	36	100
173	DBA	Levan & Hauschka 1952	Philadelphia	36–78	59–73	12	42	28	50
174		Feldman & Sachs, 1958		39–44	-	6	42	54	50
175	Leuk 1210	Levan, unpubl	SKI, Friend	41–46	-	6	43	45	100
176	Asc Hepat	Sato et al., 1956	134	42–93, 143	53–86	16	46	25	100
177			129F	43–93, 140	49–85	13	45	29	100
178			129P	43–91	48–79	11	45	57	100
179	SJA	E Klein, 1955		66–77	-	12	72	31	100
180		Ising, 1958		59–79	-	12	72	26	174
181			in hamster	62–79	-	14	72	31	199
182			in cortisone hamster	61–78	-	15	70	25	175
183	MC1M	Levan & Hauschka 1953	Philadelphia	55–270	145–175	36	70	14	119
184	MC1A			76–168 225	95–149	15	80	31	39

Rat ascites tumors (Table 7) have been investigated mainly by Japanese workers, pioneer work in the chromosome study of cancer having been carried out on the Yoshida sarcoma by Makino and his

TABLE 7. Ascites Tumors of the Rat (Cases 185 to 223)

1. No	2. Tumor	3. Reference	5. Tumor Line	6. Variation Extremes	8. Different Numbers	9. s	10. % Cells with s	12. Number of Cells
185	Yoshida	Sato, 1950	Original	29–102	26	42	11	100
186		Makino & Kanô, 1951	〃	22–80	45	40	13	620
187		Nakahara, 1952	in mouse	35–42	8	41	43	37
188		Hirono & Yokoyama, 1955	Original	22–93	40	38, 39	7	198
189			Resistant to Nitrogen Mustard	25–94	37	37	10	198
190		Tjio & Levan, 1956b	Stockholm	39–43	5	40	96	407
191		Ising, 1958	〃	36–41	5	40	44	45
192			in mouse	38–42	5	40	53	40
193			in hamster	39–41	3	39	59	17
194		Makino & Sasaki, 1958	A	36–44	7	39	67	125
195			B	38–43	6	40	61	101
196			C	36–42	7	38	42	180
197			D	35–42	8	39	43	229
198	MTK I	Tanaka & Kanô, 1951		26–87	30	41	18	222
199	MTK II	Tonomura, 1954		33–46	14	40	26	158
200		Ohnuki, 1956		35–42	7	39	57	63
201			in mouse	36–42	7	39	60	50
202	MTK III	Umetani, 1953		35–45	-	40	-	-

203		Makino, 1957		40–44	5	41	72	47
204	Hirosaki	Makino & Kanō, 1953	Original	36–42	7	39	31	112
205			Clone B-H	36–39	-	37	-	-
206			C-H	37–41	-	39	-	-
207			D-H	35–39	-	37	-	-
208			E-H	36–40	-	38	-	-
209	AH 130	Yoshida, 1957		29–>81	15	43	23	100
210		Levan, unpubl.	from Koprowski	44–51	8	50	32	57
211	AH 7974	Yoshida, 1957		36–>81	16	49	28	100
212	AH 601			40–>81	>22	67	15	100
213	AH 602			53–>81	>22	67	13	100
214	AH 66			62–81	18	70	13	100
215	MTK IV	Makino, 1956	Gen. 30–50	62–68	-	67	-	-
216		Ohnuki, 1956	Gen. 87	58–62	5	60	57	23
217			in mouse	59–67	5	60	50	20
218	Hirosaki	Makino, 1957		69–75	-	74	-	-
219	Usibuchi	Makino, 1957		65–80	-	70–76	-	-
220	Takizawa	Yosida, 1954a		34–80	13	75, 76	19	21
221	Takeda	Yosida, 1954c		36–104	17	84	15	26
222		Makino, 1956		37–163	-	73–87	-	-
223	Watanabe	Watanabe & Tonomura, 1956		±20– ±300	-	80–90	-	-

TABLE 7. Ascites Tumors of the Rat (Cases 185 to 223)

1. No	2. Tumor	3. Reference	5. Tumor Line	6. Variation Extremes	8. Different Numbers	9. s	10. % Cells with s	12. Number of Cells
185	Yoshida	Sato, 1950	Original	29–102	26	42	11	100
186		Makino & Kanô, 1951	"	22–80	45	40	13	620
187		Nakahara, 1952	in mouse	35–42	8	41	43	37
188		Hirono & Yokoyama, 1955	Original	22–93	40	38, 39	7	198
189			Resistant to Nitrogen Mustard	25–94	37	37	10	198
190		Tjio & Levan, 1956b	Stockholm	39–43	5	40	96	407
191		Ising, 1958	"	36–41	5	40	44	45
192			in mouse	38–42	5	40	53	40
193			in hamster	39–41	3	39	59	17
194		Makino & Sasaki, 1958	A	36–44	7	39	67	125
195			B	38–43	6	40	61	101
196			C	36–42	7	38	42	180
197			D	35–42	8	39	43	229
198	MTK I	Tanaka & Kanô, 1951		26–87	30	41	18	222
199	MTK II	Tonomura, 1954		33–46	14	40	26	158
200		Ohnuki, 1956		35–42	7	39	57	63
201			in mouse	36–42	7	39	60	50
202	MTK III	Umetani, 1953		35–45	-	40	-	-

school. A whole group of tumors, viz., Yoshida, MTK I, II, III, Hirosaki diploid, all having hypodiploid stemlines between 38 and 42, exists in a great number of sublines and clones (Cases 185–208). Since they all have a very specific karyotype with big metacentric chromosomes, they probably are of common origin. The Japanese rat ascites tumors were recently reviewed by Makino (1957). Broadly speaking, their chromosome variation and stemline numbers are in agreement with what is known for mouse tumors. Their stemlines are distributed between $2x$ and $4x$ with some favoring of hypotetraploid numbers.

3. Cell Strains

Serially carried ascites tumors have a counterpart among tissue cultures in the continuously growing cell strains. In both materials the adaptation to the environment is good. What makes the cell strains *especially* relevant in the present connection is the fact that cell strains established from normal cells tend to attain malignant properties, behaving as tumors if inoculated into compatible hosts. The adaptation to continuous life *in vitro* often includes capacity for cancer growth.

In the mouse, the oldest and most intensely studied cell strain is the L strain, originally developed by Earle from subcutaneous adipose tissue of C3H mice It has been divided, by clonal and few-cell isolation, by growth in different media, etc., into a great number of sublines. During the last year three sets of sublines, grown in Bethesda, Houston, and Toronto, have been subjected to chromosome analysis by Chu, Sanford, and Earle (1958), Hsu and Klatt (1958), and Rothfels *et al.* (in press), respectively (Table 8).

As seen from Table 8, essential similarities in chromosome picture exist between ascites tumors and cell strains: The chromosome number variation around a modal number, and the heteroploid location of the stemline Since, however, the cell strains are adaptations to a different kind of environment than ascites tumors, differences in the resulting genotypes should be anticipated. The L strain differs from mouse ascites tumors in two obvious ways: (1) It has a regular and, especially in the Houston and Toronto materials, high frequency of metacentrics. Although ascites tumors are known to hold metacentrics occasionally, their frequency per stemline is never high The L sublines, however, may have as many as 22 metacentrics per cell average. (2) The level of stemline number in the Houston and Toronto materials is constantly above 80. If chromosome arms are counted as units, the following stemline numbers are found:

TABLE 8. Chromosome Numbers of Murine Cell Strains (Cases 224 to 241)

1 No	2 Cell Strain	3 Reference	5 Tissue of Origin	6 Variation Extremes Chromosomes (Arms)	8 Different Numbers	9 m	10 % Cells with m	11 Number of Metacentrics	12. Number of Cells
224	NCTC 1742	Chu, Sanford, & Earle, 1958	Subcutaneous adipose tissue	50–77, 91–170 (53–80, 94–173)	18	56 (59)	20	3	51
225	2672			43–75, 80–120 (47–79, 84–124)	23	51 (55)	7	4	70
226	2472			44–61, 91–120 (46–63, 93–123)	16	55 (57)	14	2	42
227	2649			42–53, 81–90 (48–59, 87–96)	12	49 (55)	27	6	45
228	2673			41–62, 81–130 (42–63, 82–131)	22	54 (55)	16	1	68
229	2445			46–77, 101–110, 181–190 (48–79, 103–112, 183–192)	17	53 (55)	22	2	58
230	2555			62–78, 190 (73–89, 200)	14	69 (80)	28	11	50
231	2470			46–65, 81–110 (49–67, 83–112)	12	52 (54)	22	2	27
232	MCl	Hsu & Klatt, 1958	Bone marrow	75–82 (91–100)	8 (10)	78 (95)	30 (32)	17	50
233	CFI-C 9		Bone marrow	68–98 (83–114)	15 (17)	82 (97)	22 (24)	16	50
234	NCTC cl 929		Subcutaneous adipose tissue	69–76 (89–98)	8 (10)	74 (94)	34 (32)	22	50
235	LPS5		Substrain of cl 929	63–70 (83–92)	7 (10)	69 (89)	30 (20)	19	50
236	LPS5 N2		Substrain of LPS5	64–75 (82–93)	10 (11)	68 (88)	32 (20)	18	50
237	2-2	Rothfels *et al*, in press	Subcutaneous adipose tissue	59–71 (75–90)	13 (15)	69 (86)	17 (19)	18	100
238	3-3			64–72 (81–90)	8 (10)	67 (85)	22 (22)	17	50
239	MsK 1			65–85 (82–102)	8 (7)	81 (98)	22 (33)	17	18
240	MsK 2			75–85 (92–102)	7 (8)	82 (99)	35 (32)	17	31
241	MsK 3			49–87 (59–104)	10 (10)	82 (99)	39 (39)	16	31

school. A whole group of tumors, viz., Yoshida, MTK I, II, III, Hirosaki diploid, all having hypodiploid stemlines between 38 and 42, exists in a great number of sublines and clones (Cases 185–208). Since they all have a very specific karyotype with big metacentric chromosomes, they probably are of common origin. The Japanese rat ascites tumors were recently reviewed by Makino (1957). Broadly speaking, their chromosome variation and stemline numbers are in agreement with what is known for mouse tumors. Their stemlines are distributed between $2x$ and $4x$ with some favoring of hypotetraploid numbers

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Strain	*54*	*55*	*57*	*59*	*80*	*85*	*86*	*88*	*89*	*94*	*95*	*97*	*98*	*99*	*Mean*
Bethesda	1	4	1	1	1	–	–	–	–	–	–	–	–	–	59
Houston, Toronto	–	–	–	–	–	1	1	1	1	1	1	1	1	2	93

No spontaneous mouse ascites tumor with stemline numbers above 80 is known, the only ones on record being Ehrlich clone 1 and EL88.

Table 9 surveys some 40 cases of human cell strains, half of them being of normal and half of cancer origin. These two groups are similar in chromosome behavior; any differences originally existing have become leveled during the *in vitro* life. A number of cell strains (cases marked "Levan, unpublished," Table 9) were grown during 1957 at the Sloan-Kettering Institute by Dr. Alice Moore, who kindly gave them over to me for chromosome study. In these cases sometimes only a single cell or a few cells were analyzable, but, even so, complete idiogram analysis was made.

It seems that human cell strains on an average have a somewhat higher chromosome number level than human tumors (cf. Ising and Levan, 1957). The development of a cell strain out of a primary culture tends to shift the stemline number over into the triploid-tetraploid region. The commonest stemline numbers in human tumors are diploid-triploid. Also, the lower limit for stemline numbers in cell strains is as high as 65, while several hypodiploid human tumors exist. The upper limit for a cell strain is 133; so far, no human tumor above 92 is known.

Gey's HeLa strain, being a counterpart in man to Earle's L strain of the mouse, exists in many sublines. The HeLa strains of Table 9 (Cases 266–277) contain the following stemline numbers:

68	*70*	*73*	*74*	*76*	*78*	*79*	*82*	*Mean*
1	1	1	1	1	3	1	3	77

As should be expected, each of these cell lines has its individual chromosome pattern. Thus, one of them (Case 273) is remarkable for its chromosome stability, only four numbers being recorded, and 91 per cent of all cells belonging to the stemline. Another subline (Case 272) showed the opposite extremity with 22 different numbers and 21 per cent of the cells belonging to the stemline.

Since identical environment can never be reproduced and evolution proceeds over a great number of unpredictable steps, it may be assumed that the division into sublines of any cell strain, however homogeneous, will always predispose to cytogenetic differentiation.

This involves a serious practical problem: Will it be possible to maintain a standardized type of such a cell strain as the L strain or the HeLa? How can we know that a strain now under analysis will be the same on a later occasion? Karyotype control is a means of identification that may help in detecting gross stemline shifts or crude contaminations. However, a strain may undergo extensive genetic changes without this being apparent in the karyotype.

4. Cancer Progression as Genetic Adaptation

The chromosomal evidence now briefly presented points decidedly in one direction. The stemlines of old transplantable tumors, as well as of cell strains, have developed from the normal genotype by mutative adaptation. This is a process that is never concluded, new adaptations always being under way. The mammalian cell exhibits a surprising latitude of adaptability: It will cast off from its original co-ordination in the tissue and start out on its own individual evolution. By random genetic changes, a certainly very minor part of which may be visible in the microscope as chromosome number variation, gross structural variation, and cryptostructural variation, the cell population becomes heterogeneous and thus responsive to selection. By the accumulation in cell lineages of mutations, favorable under the prevailing conditions, a good viability is obtained eventually under life habits, foreign to the original cell. The development goes toward an ever more independent way of life.

Thus, the cytogenetic mechanisms underlying tumor progression are familiar, in principle. Their similarity on the cellular level to evolutionary changes on the organism level has often been pointed out. The next, and more important, question is whether mechanisms of similar kind are active at the first deviation from normality, when an ordinary soma cell is started off on its way toward malignancy.

5. Cancerogenesis and Early Cancer Stages

Nothing is known about chromosome behavior at cancerogenesis *in situ*, although something may be inferred from the study of early stages of primary cancers. In an indirect way, however, some information may be obtained from the study of cellular adaptations in tissue culture.

The first investigation of this kind was by Hsu, Pomerat, and Moorhead (1957), on the development of the human cell strain Mayes. This started out as a culture of normal cells from the synovial lining of the leg (Table 2, Case 45). Up to the fifth passage chromosome

Strain	*54*	*55*	*57*	*59*	*80*	*85*	*86*	*88*	*89*	*91*	*95*	*97*	*98*	*99*	*Mean*
Bethesda	1	4	1	1	1	–	–	–	–	–	–	–	–	–	59
Houston, Toronto	–	–	–	–	–	1	1	1	1	1	1	1	1	2	93

No spontaneous mouse ascites tumor with stemline numbers above 80 is known, the only ones on record being Ehrlich clone 1 and EL88.

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68	*70*	*73*	*74*	*76*	*78*	*79*	*82*	*Mean*
1	1	1	1	1	3	1	3	77

As should be expected, each of these cell lines has its individual chromosome pattern. Thus, one of them (Case 273) is remarkable for its chromosome stability, only four numbers being recorded, and 91 per cent of all cells belonging to the stemline. Another subline (Case 272) showed the opposite extremity with 22 different numbers and 21 per cent of the cells belonging to the stemline

Since identical environment can never be reproduced and evolution proceeds over a great number of unpredictable steps, it may be assumed that the division into sublines of any cell strain, however homogeneous, will always predispose to cytogenetic differentiation.

261	J 96	*Hsu & Moorhead* 1957		*41–100, 150*	-	69	-	20
262		Levan, *unpubl*		*61–67*	5	-	-	9
263	HEp2 SK1	*Levan*, 1956b	*Epidermoid carcinoma*	*74–90*	-	-	-	6
264		unpubl.		*66–83*	14	76	22	23
265	HeLa	Hsu, 1954	Cervical carcinoma	71–200	-	81–90	-	*100*
266		Chu & *Giles*, 1958	*Clone from HeLa*	64–89	17	82	28	57
267	S1			*70–84*	12	78	59	103
268	S3	Hsu & Moorhead, *1957*		*61*–95, 150	-	82	-	50
269		Chu & Giles, *1958*		70–82, 113, 150	12	78	62	*100*
270	S3R3		Clone from S3	72–80, 126	9	74	70	100
271	S3R3A1			62–75	*10*	68	55	101
272	S3R3E1			58–105	22	82	21	100
273	*S3-9*	Tjio & Puck, 1958a		71–74	4	73	91	108
274	*S/NDV*		*Clone* from HeLa	75–82	8	78	82	139
275	S3 St1	Vogt, 1958	*Clone* from HeLa S3	77–82	6	79	43	60
276	F8			67–74	8	70	51	52
277	F9			74–81	7	76	47	55
278	Maben	*Hsu & Moorhead*, 1957	Adeno*carcinoma of* the lung	56–100, 150	-	82	-	50
279	KB		Epider*moid* carcinoma	61–95, *141–150*	-	83	-	50
280	*H Ep.1* SK1	Levan, 1956b		91	-	-	-	1
281		unpubl.		85–96	-	±90	-	7
282	Fjelde	Norryd, *1959*		*68*–76	9	72	40	50
283	HS1 SK1	Levan, *unpubl.*		114–120	4	±118	-	4

TABLE 9. Chromosome Numbers of Human Cell Strains (Cases 242 to 260)

1. No	2. Cell Strain	3. Reference	5. Tissue of Origin	6. Variation Patterns	8. Different Numbers	9. m	10. % Cells with m	11. Number of Cells
242	McCoy	Hsu & Moorhead, 1957	Synovial fluid, knee joint	36–120, 150	*	67	*	100
243	FL		Sole of the foot	56–80, 106–135	*	69	*	50
244		Levan, unpubl.		64–67	3	166	*	4
245	S-12-1	Syverton, 1957	Esophageal epithelium	65–79	*	70	*	8
246	Clonal 1a			65–79	*	70	*	17
247	Bergman	Norryd, 1959	Parotid	70–82	13	73, 79	24, 18	50
248	Pugh		Amnion	72–82	10	76	36	50
249		Levan, unpubl.		70–88	15	76	26	47
250	Chang	Levan, 1956b	Liver epithelium	75	*	*	*	1
251		Hsu & Moorhead, 1957		36–110, 141–150	*	77	*	100
252		Levan, unpubl.		76–84	6	81–82	21	11
253	Minnesota EE 2	Syverton, 1957		58–78	*	75	*	18
254	Chang	Levan, 1956b	Conjunctiva	70–77	*	*	*	4
255		Hsu & Moorhead, 1957		46–115, 150	*	82	*	50
256		Syverton, 1957		64–81	*	80	*	16
257		Levan, unpubl.		75–84	7	100	*	11
258	Kidney		Kidney	114–120	4	118	-	4
259	Meyer, Pass 9	Hsu, Pomerat, & Moorhead, 1957	Synovial lining	55–70, 85–115, 200	*	111		11
260	J111	Levan, unpubl.	Chronic myeloid leukemia	56–70, 114	7	65	14	14

262		Levan, unpubl		[illegible]	-			
263	HEp2 SK1	Levan, 1956b	Epidermoid carcinoma	74-80	-	-	-	6
264		unpubl		66-83	14	76	22	23
265	HeLa	Hsu, 1954	Cervical carcinoma	71-200	-	81-90	-	100
266		Chu & Giles, 1958		64-89	17	82	28	57
267	S1		Clone from HeLa	70-84	12	78	59	103
268	S3	Hsu & Moorhead, 1957		61-95, 150	-	82	-	50
269		Chu & Giles, 1958		70-82, 113, 150	12	78	62	100
270	S3R3		Clone from S3	72-80, 126	9	74	70	100
271	S3R3A1			62-75	10	68	55	101
272	S3R3E1			58-105	22	82	21	100
273	S3-9	Tjio & Puck, 1958a		71-74	4	73	91	108
274	S/NDV		Clone from HeLa	75-82	8	78	82	139
275	S3 St1	Vogt, 1958	Clone from HeLa S3	77-82	6	79	43	60
276	F8			67-74	8	70	51	52
277	F9			74-81	7	76	47	55
278	Maben	Hsu & Moorhead, 1957	Adenocarcinoma of the lung	56-100, 150	-	82	-	50
279	KB		Epidermoid carcinoma	61-95, 141-150	-	83	-	50
280	H.Ep.1 SK1	Levan, 1956b		91	-	-	-	1
281		unpubl.		85-96	-	±90	-	7
282	Fjelde	Norryd, 1959		68-76	9	72	40	50
283	HS1 SK1	Levan, unpubl.		114-120	4	±118	-	4

TABLE 9. Chromosome Numbers of Human Cell Strains (Cases 242 to 283)

1. No	2. Cell Strain	3. Reference	5. Tissue of Origin	6. Variation Extremes	8. Different Numbers	9 s	10. % Cells with s	12. Number of Cells
242	McCoy	Hsu & Moorhead, 1957	Synovial fluid, knee joint	36–120, 150	-	67	-	100
243	FF		Sole of the foot	56–80, 106–135	-	69	-	58
244		Levan, unpubl.		64–67	3	:66	-	3
245	5-12-1	Syverton, 1957	Esophageal epithelium	65–79	-	70	-	8
246	Clonal 1a			65–79	-	70	-	17
247	Bergman	Norryd, 1959	Parotid	70–82	13	73, 79	24, 18	50
248	Fogh		Amnion	72–82	10	76	36	50
249		Levan, unpubl.		70–88	15	76	26	47
250	Chang	Levan, 1956b	Liver epithelium	75	-	-	-	1
251		Hsu & Moorhead, 1957		36–110, 141–150	-	77	-	100
252		Levan, unpubl.		76–84	6	81–82	23	13
253	Minnesota 6-6-2	Syverton, 1957		58–78	-	75	-	18
254	Chang	Levan, 1956b	Conjunctiva	70–77	-	-	-	4
255		Hsu & Moorhead, 1957		46–135, 150	-	82	-	50
256		Syverton, 1957		63–81	-	80	-	16
257		Levan, unpubl		75–83	7	:80	-	11
258	Kidney		Kidney	114–120	4	:118	-	4
259	Mayes, Pass 9	Hsu, Pomerat, & Moorhead, 1957	Synovial lining	55–70, 85–145, 200	-	133	-	43
260	J 111	Levan, unpubl.	Monocytic leukemia	56–70, 114	7	65	33	

TABLE 10 Chromosome Numbers of Embryonic Skin Tissue of the Mouse during Serial Passage *in vitro* Resulting in the Formation of a Malignant Cell Strain (*Levan & Biesele, 1958*) (Cases 284 to 292)

1. No	5. Passage No	6. Variation Extremes	8. Different Numbers	9 <u>s</u>	10. % Cells with <u>s</u>	12. Number of Cells
284	1, 2	39–160 (39–42, 73–83, 160)	15	40	55	150
285	4	39–160 (39–47, 66–81, 160)	14	80	19	31
286	9–11	40–320 (40–41, 66–110, 138, 160, 260, 320)	20	80	45	103
287	16–18	53–320 (53–93, 130–150, 320)	29	None	-	85
288	19–21	58–320 (58–95, 130–160, 320)	23	75	15	62
289	23	58–140 (58–82, 130–140)	21	68	11	102
290	35	69–84	13	74–75	25	40
291	36	71–83	11	75	25	60
292	22 x)	60–68	9	64	20	25

x) After passage through mouse as spindle cell sarcoma

behavior was mainly normal. In passage 8 a very wide chromosome number variation was noted with no predominant stemline, and in passage 9 a new stemline at the high level of 133 started materializing (Table 9, Case 259). A similar step from normality to a highly increased stemline number was observed by Levan (unpublished) in a culture of normal human kidney, grown by Miss Frances Mottram at the Sloan-Kettering Institute (Table 2, Case 68), and a third case was Miss Diamond's human amnion culture, mentioned previously, in which at passage 4 one culture tube contained a majority of cells with chromosome numbers around 70 (Table 2, Case 67).

In one experimental series with embryonic mouse skin (Levan and Biesele, 1958) the chromosomes were observed in many samples from the first passage until the establishment of a cell strain, later on acquiring malignancy. This entire development was characterized by a continuous chromosome number variation (Table 10). As early as in passage 4 (Case 285) the tetraploid number prevailed. The further development passed stages with very wide variation, sometimes with no definite stemline mode (Case 287). Gradually the variation gathered at the hypotetraploid region, two stemline equilibria forming, one at 64 and the other at 75. The former one (Case 292) was characteristic of the cell strain after passage as a spindle cell sarcoma through mice, the latter (Cases 290–291) occurred after permanent carriage *in vitro*. From the very start this numerical variation was associated with frequent structural changes, in some cases affecting as many as 20 per cent of the anaphases.

There is little doubt in this case that cytogenetic variation is the main factor responsible for the change of the normal mouse skin cells into a cell strain with malignant potential. The starting point of this development is evidently the change in environment at the explantation of the cells. If any conclusions can be drawn from this experiment as to cancerogenesis in the body, it seems that cancer development should be enhanced by: (1) Chromosomal and genic variation; (2) High mitotic rate, bringing about somatic segregations; (3) Change in cellular environment, placing the normal genotype at disadvantage, thus favoring experimentation with genotypes of increased independence. Whether genotypic changes alone are enough to induce this sequence of events, as assumed by the somatic mutation theory, is still an open question Some guidance on this point may be obtained from chromosome analysis of early stages of primary cancers. Although little is known in this field, a few recent investigations have demonstrated conclusively that essentially different chro-

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284	1, 2	39–160 (39–42, 73–83, 160)	15	40	55	150
285	4	39–160 (39–47, 66–81, 160)	14	80	19	31
286	9–11	40–320 (40–41, 66–110, 138, 160, 260, 320)	20	80	45	103
287	16–18	53–320 (53–93, 130–150, 320)	29	None	-	85
288	19–21	58–320 (58–95, 130–160, 320)	23	75	15	62
289	23	58–140 (58–82, 130–140)	21	68	11	102
290	35	69–84	13	74–75	25	40
291	36	71–83	11	75	25	60
292	22 x)	60–68	9	64	20	25

x) After passage through mouse as spindle cell sarcoma

mosome mechanisms may be involved in the early development of different types of tumors.

Cancerogenesis by mutative adaptation should be expected among cancers induced by mutagenic agents. Some recent studies seem to support this notion. Ford and collaborators (1957, 1958), working with a number of primary mouse leukemias, reticulosarcomas, etc., often induced by x-irradiation, observed a wider chromosome number variation than in normal tissues. Each tumor had its own stemline karyotype with chromosome numbers between 40 and 44. A similar chromosomal background was reported by Bayreuther and E. Klein (1958) for a group of sublines of the methylcholanthrene-

TABLE 11. Chromosome Numbers of Primary Virus-Induced Tumors (Cases 293 to 312)

1 No	3. Reference	5 Tumor Type	6 Variation Extremes	8 Different Numbers	9. n	10. % Cells with n	11 Number … Cells
293	Tjio & Östergren, 1958	Mammary carcinoma in dba	39–41	3	40	95	169
294			39–41	3	40	90	167
295			39–41, 80	4	40	90	150
296			39–41, 80	4	40	95	131
297			39–41, 80	4	40	89	159
298			39–41, 80	4	40	96	109
299			39–41, 80	4	40	95	139
300			39–41, 80	4	40	97	125
301			39–41	3	40	98	144
302			40–41	2	40	99	121
303			39–41	3	40	97	109
304			40, 80	2	40, 80	58, 42	145
305	Tjio & Östergren, 1958	Mammary carcinoma in C3H	39–41	3	40	95	162
306			39–41, 80	4	40	91	149
307			40	1	40	100	120
308			39	1	39	100	114
309			40–41	2	40	99	122
310			39–41, 80	4	40	94	150
311			78–81	4	80	92	120
312	Levan, unpubl	Friend s leukemia	39–41, 80	4	40	77	171

induced mouse sarcoma MSWB. In these cases the stemline karyotype may well represent early steps in an evolution by mutative adaptation.

Recently Tjio and Östergren (1958) at our laboratory studied some 20 milk factor-dependent primary mammary carcinomas of dba and C3H mice. The chromosome conditions at early stages of these tumors were entirely different from the above-mentioned tumors and quite incompatible with a cancerogenesis by extensive structural reorganization. It is shown in Table 11 that among 19 individual tumors, 16 had a stemline with the normal diploid number 40, one had 39 chromosomes (Case 308), one had 80 (Case 311), one finally had two stemlines, 40 and 80 (Case 304). The chromosome stability was striking, the maximal variability around the stemline number being ±1 chromosome. In two tumors, just one number was found. No structural changes were seen.

Tjio and Östergren made the suggestive discovery that, in all tumors studied, except for the one with 39 chromosomes, one chromosome per diploid set had become heteropyknotic. It was isopyknotic at metaphase, as compared with the other chromosomes, but at all other stages it exhibited positive heteropyknosis The authors, naturally, associate this remarkable phenomenon with the fact that the viruslike milk factor is involved in induction of the tumors, suggesting a *direct* influence of the virus particles on one specific chromosome, thus, a kind of virus infection of one chromosome.

The only other virus-induced tumor so far studied is the leukemia of Dr Friend. Preliminary results (Levan, unpublished) showed it to have a limited numerical variation (Case 312). The suspected presence of minor structural alterations has to be confirmed further.

6 Concluding Remarks

The present survey of chromosome numbers in normal tissues, ascites tumors, and cell strains has led to some conclusions as to cytogenetic events in cells and tissues active during their adaptation to new life habits. These cytogenetic events are more familiar on the level of organisms and populations of organisms, that is, during natural evolution

The normal somatic karyotype of the species is the starting point for the cellular evolution Ascites tumors and cell strains are advanced stages of evolution toward two different types of environment. Common to both of them is the acquisition of increased independence for the individual cell. Karyotypic developments prior to these

mosome mechanisms may be involved in the early development of different types of tumors.

Cancerogenesis by mutative adaptation should be expected among cancers induced by mutagenic agents. Some recent studies seem to support this notion. Ford and collaborators (1957, 1958), working with a number of primary mouse leukemias, reticulosarcomas, etc., often induced by x-irradiation, observed a wider chromosome number variation than in normal tissues. Each tumor had its own stemline karyotype with chromosome numbers between 40 and 44. A similar chromosomal background was reported by Bayreuther and E. Klein (1958) for a group of sublines of the methylcholanthrene-

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294			39–41	3	40	90	167
295			39–41, 80	4	40	90	150
296			39–41, 80	4	40	95	131
297			39–41, 80	4	40	89	159
298			39–41, 80	4	40	96	109
299			39–41, 80	4	40	95	139
300			39–41, 80	4	40	97	125
301			39–41	3	40	98	144
302			40–41	2	40	99	121
303			39–41	3	40	97	108
304			40, 80	2	40, 80	58, 42	145
305	Tjio & Östergren, 1958	Mammary carcinoma in C3H	39–41	3	40	95	162
306			39–41, 80	4	40	91	138
307			40	1	40	100	120
308			39	1	39	100	134
309			40–41	2	40	99	122
310			39–41, 80	4	40	94	150
311			78–81	4	80	92	120
312	Levan, unpubl	Friend s leukemia	39–41, 80	4	40	77	171

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advanced stages are still only little known, the analysis of early cancer stages being especially urgent.

It seems that the formation of cell strains with malignant properties, that is, cancerogenesis *in vitro*, may proceed from beginning to end by mutative variation. Similar cytogenetic mechanisms may account for cancer progression, and possibly for cancerogenesis by mutagenic agents. However, results from viral cancers indicate that chromosomes are involved in this kind of cancerogenesis in an essentially different way.

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Genetics of *in vitro* Cells

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For the past two decades microorganisms have dominated the scene of research in biochemical genetics. This has been due primarily to the fact that nutritional systems of Neurospora, yeasts, and a number of bacteria require rather simple growth media whose ingredients are principally inorganic. Another reason for the popularity of microbes in biochemical genetics has been that they grow and multiply at a terrific rate, unequaled by other organisms.

During the past few years, mammalian cells have emerged as one of the widely used materials for biological and medical research. Cells in ascites and in tissue culture, especially the latter, can be handled in some ways like microbes. Investigators utilizing the ascites system are relatively handicapped in studying metabolism because their material is cultivated *in vivo* and is thereby involved in a complex physiological system. Recently, however, comparative biochemical analyses have been started on the variation of the free amino acid content and of several enzymatic activities between diploid and tetraploid tumor cells (Kit, Fiscus, Graham, and Gross, 1959).

Several new advances in cell culture have helped to draw the attention of workers in the fields of genetics and biochemistry to *in vitro* cells: 1. Many cell strains are readily available to provide an endless supply of material; 2. improvements in culture techniques have made routine handling a relatively easy task; 3. single cells can be isolated to produce clonal derivatives; and 4. improvements in

nutritional studies already have produced several chemically defined media so that natural products such as serum, plasma, and embryo extract can be eliminated or reduced to the minimum.

It is quite reasonable to assume that mutations may arise in populations of mammalian cell cultures. There have been a number of reports in the literature describing sudden or dramatic morphological changes of *in vitro* cells. A part of them were proved to be contaminants from other strains (Rothfels *et al.*, in press). Investigators working with cell lines also are acquainted with the fact that the nutritional state of a culture may affect the appearance of the cells Nevertheless, this does not prove that morphological "mutations" have never occurred.

Conventional methods of obtaining metabolic mutants from microbial populations can be applied to mammalian cells for selection of special physiological traits. The fact that mammalian cells cannot be grown in an inorganic medium makes it highly impracticable to select mutants that are metabolite dependent. The alternative would be the selection of cells that are independent of certain metabolic requirements or that are resistant to a certain environment.

In their attempts to subject L cells to a chemically defined medium, McQuilkin, Evans, and Earle (1957) treated a population of clone 929 which had been grown in serum medium with a special medium devoid of natural proteins. Practically all the cells in the container succumbed. The authors later found a single colony that probably developed from a single cell which was able to propagate itself utilizing the ingredients of the medium for growth. Such cases strongly suggest the existence of "biochemical mutations." It is beyond doubt that such investigations would stimulate great progress toward an understanding of the nutritional requirements of mammalian cells. Metabolic processes involved in this type of mutation, however, are probably too complex for profitable analysis of any particular pathway.

Similar problems of complex biochemical patterns exist in the cases of L cells that are resistant to toxic sera from schizophrenic human patients (Fedoroff and Cook, 1959) and of HeLa cells resistant to polio virus (Vogt, 1958) and to Newcastle virus (Puck and Cieciura, 1958).

More specific, perhaps, are the "mutants" selected with vitamins (Haff and Swim, 1957), with carbohydrates (Chang, 1957), and with asparagine requirements (McCoy *et al.*, 1959). These examples, together with the method developed by Moser (1958) for

increasing mutation rates by combining irradiation of plated cells with a treatment in a specific medium, paved the way for induction of mutants and quantitative analysis of biochemical activities in mammalian cells.

It must be kept in mind that cells in continuous culture are not necessarily normal. It has been well documented that most cell lines are heteroploid and are cytologically polymorphic. Even without considering the possibility of mutations, which undoubtedly would have occurred repeatedly in the history of a cell line, reshuffling of existing chromosomes should be sufficient to alter the normal balance in the genetic makeup of the cells and in turn to alter certain metabolic phenotypes. Therefore, a metabolic variant isolated from *in vitro* cells may or may not be due to a mutation in the strict sense. In spite of the handicap that somatic cells in culture lack sexual recombination for classical genetic studies, the mammalian cells possess an advantage over most microbes; namely, their discernible chromosomes, many of which are good markers.

We had intended to try to correlate metabolic abilities of "mutants" with their cytological features. Our interests in the relationships between chromosome constitution and glycolytic activities of cell lines (unpublished data) stimulated us to select metabolic variants involving carbohydrate utilization. In the summer of 1957 Dr. R. S Chang of the Harvard School of Medicine contacted one of us (Hsu) to discuss a possible co-operative project to study the chromosomes of certain human cell lines that were able to utilize xylose instead of glucose as the carbon source. The co-operation was not found feasible because of our geographical separation and the difficulty in analyzing human chromosomes. However, Dr. Chang urged us to select similar lines with cytologically more favorable materials for such an approach Beginning in November of 1957, we selected strain L-P55 as our material for such a study. Since it was desirable to select variants with qualitative as well as quantitative changes, and since it has been shown that d-galactose is utilized inefficiently and that d-xylose is not utilized (cf. Waymouth, 1957), these two sugars were chosen. The ability of cells to survive for short periods in the presence of these sugars was not sufficient. The cells must be able to utilize the carbohydrates supplied in replacement of glucose over a prolonged period of cultivation and be able to produce an expanding population. The present article reports our preliminary results in the "sugar lines," which may serve as a model for future exploration of biochemical genetics of mammalian cells

MATERIALS AND METHODS

Strain L-P55, a subline of clone 929, strain L, and its derivative "mutant" lines were used throughout, in the present study. These cells were cultivated in Eagle's basic medium in which Eagle's salt solution was replaced with Gey's salt solution. Unless otherwise stated in the text, all media contained 5 per cent nondialyzed horse serum. For strain L-P55, glucose was supplied through Gey's solution. For the mutant strains, however, a basic medium was prepared without carbohydrates save that indigenous to horse serum. Special carbohydrates were added to the basic medium for the special strains: d-galactose or d-xylose, 5 millimoles each.

During the selective procedures and up to the time when growth experiments were initiated, Difco horse serum had been employed exclusively. Repeated loss of cell lines due to toxicity of some of the sera necessitated our subsequent change to a product of the Colorado Serum Company, which had pretested the sera for toxicity according to Puck's procedure of cloning.

All media were adjusted to approximately pH 7.4 prior to use. Incubation of cultures was at 35° to 36° C. The medium required to cover the cell sheets was maintained at approximately a 13:1 air-fluid ratio in all vessel types. Generally the growth medium was changed twice weekly, and subcultures were made approximately every two weeks. Three different types of culture vessels were employed in our experiments. The T-series flasks were used during the production of the sugar lines because of their excellent optical quality for microscopic inspection However, when cell lines were considered established, Blake bottles were then used for routine propagation. For growth curve assays, the cells were seeded in new, unwashed, and unsterilized screw-capped prescription bottles (Owens Ovals). Since the bottles were capped at the factory while the glass was still hot and consequently sterile, no microbial contamination developed in the cultures. The bottles were so inexpensive that it was considered more economical to use them only once, rather than to wash and resterilize them

The following procedure for setting up growth curves was used for all strains in all media. Three days before initiation of growth assays, fresh medium was applied to stock cell cultures. Cells were harvested by replacing the medium with 5 ml of warm 0.005 per cent Bacto-trypsin in Gey's salt solution without glucose The cells were suspended rapidly with the aid of a rubber fishtail policeman, and 5 ml.

of additional Gey's solution without glucose was introduced into each bottle. The cells were pooled, centrifuged, and resuspended in 10 ml. of Gey's salt solution without glucose. Duplicate cell counts were made with the aid of a Brite Line hemacytometer (see discussion of cell counts under "Growth"), and the required number of cells for each experiment were pipetted into the specific test medium, shaken thoroughly, and dispensed into the Owens bottles. The standard initial inoculum was 5×10^5 living cells per 5 ml. of medium. The bottles were incubated without disturbance until the time for counting. No change of medium was made in any of the growth curve experiments.

At each sampling period for every growth curve, separate cell counts were made on three bottles, and the average of these counts was used in plotting the curves. The cells were suspended and counted in their growth medium. When the number of cells per bottle was, by inspection, sufficient to warrant dilution, warm trypsin solution (0.005 per cent) was added before suspending the cells. Cytological preparations were made with conventional colchicine hypotonic solution squash method.

Establishment of Cell Lines

Cultures of growing cells of L-P55 were treated with media containing either xylose, galactose, or xylose-galactose combination. Three vessels formed a set. These culture vessels were periodically examined microscopically. The majority of the cells in every flask rounded up after three to four days, and extensive cell death ensued during the first two weeks. The media were changed every seven days, and after each of the earlier changes a slight transient increase was noted, only to be followed quickly by a drop to lower numbers than the prechange levels. As a control of the growth-promoting characteristics of the basic medium, several flasks of L-P55 were set up with the basic medium only.

After four to six weeks in the xylose medium, microscopic inspection of the flasks revealed no living cells, and the cultures were presumed terminated. Later in this same period, however, a very careful microscopic examination revealed one or two small colonies of approximately a dozen cells each, in two separate flasks. These colonies slowly enlarged to macroscopic size, and a platinum wire was used to lightly scratch the colonies to dislodge some of the cells, in the hope that they might settle at other sites of the vessels. Four weeks later there were sufficient cells to make subcultures. Not all the flasks so

treated yielded variant cell lines able to utilize xylose. On the contrary, with galactose and galactose-xylose media selection was not quite as difficult, because scattered surviving cells were present at all times. After four to six weeks the number of cells in both media began to increase markedly. Eventually routine cultivation was possible for three strains of galactose cells (G_1, G_2, and G_3), three of galactose-xylose cells (GX_1, GX_2, and GX_3) and two of xylose cells (X_1 and X_2). Unfortunately, toxic horse serum eliminated strains GX_1 and X_2. Another attempt to establish xylose strains with four initial bottles yielded strain X_3. It is also of interest to mention that successful attempts were made to select cells in medium containing lactose and sucrose. These strains were also lost due to toxic horse serum.

The morphology of the cells of these sugar strains was generally the same as that of their parental line. There was, however, a noticeably greater proportion of giant cells in all the derived strains, and there was a distinct tendency with the xylose strains to assume a slightly epithelial-like appearance. The growth rate of these strains on their specific sugars was slower than that of the parental line in glucose medium; but among the "mutant" lines, the fastest growers were the GX strains, followed by the G strains, and trailing by a considerable distance were the X strains. However, little difference could be noted in their growth rates on glucose when they were regrown in glucose medium. No reselection was necessary when the cell strains were returned to their respective media after two weeks' residence in glucose medium. The sugar strains in their specific media developed considerably less acidity than in glucose medium.

In order to eliminate the possibility that growth of the "mutant" cells was not due merely to the utilization of glucose supplied by horse serum, experiments were conducted with the following results:

1. The average glucose content of horse serum was 80 mg%. So the glucose content in medium carbohydrate-free except for that supplied by horse serum was 4 mg% in the 20 ml. amount used in every medium change.

2. The cells grew in medium containing 5 per cent dialyzed horse serum (dialyzed against double-distilled water) with their respective sugars.

3. The cells grew in chemically defined medium (unpublished formula supplied by Dr. Charity Waymouth, to whom we are grateful) with their respective sugars and 0.5 per cent peptone. However, for the sake of simplicity, the stocks and experiments were all carried in nondialyzed horse serum.

Figure 1a Photomicrograph of four living (highly refractile) and four dead (dark) cells viewed with dark phase contrast optics on *Brite Line* hemacytometer

Figure 1b Same field stained with eosin but viewed with conventional light optics Living cells, light objects; dead cells, dark objects.

Growth

There are a number of accepted methods of estimating growth of a cell population. In our studies reported here we chose to use cell counts. For growing populations in which most cells are supposed to be living and healthy, cell numbers estimated by hemacytometer or by electronic cell counter should both be acceptable. When populations are subjected to unfavorable environments in which death of cells is usually common, counting without differentiating dead and viable cells generally would obscure the results. With an electronic counter this cannot be done unless the dead cells have already disintegrated when the sample is taken. With a hemacytometer, dead cells can be differentiated from living ones by the conventional eosin staining method. Our experience with phase-contrast microscopy on living cells enabled us to perform viable cell counts with a Brite Line hemacytometer and a Zeiss GF-595 phase microscope. Generally, under dark phase optics, living cells are highly refractile roundish bodies with little cellular detail; whereas dead elements are blackish and relatively flat objects with more distinct nuclei and sometimes ragged cellular outline. A cell population stained with eosin and viewed with a conventional light in comparison with the same viewed with phase microscopy showed that the two samples agree well in counts of living and dead cells. Figures 1a and 1b show, respectively, the same field of eight cells viewed: *a.* with a dark phase objective; and *b.* eosin-stained but viewed with a light objective. In each case, four were determined to be alive and four dead. Notice the differences in contrast as well as in cellular details between the two types of cells.

A growth assay was made with strain L-P55 in a medium without carbohydrates, to compare the cell counts made of living cells only and those made without differentiation. It is obvious from Figure 2 that the total cell counts obscured what had happened in a cell population which rapidly declined in the absence of carbohydrates.

Growth Responses of Cell Lines to Various Sugars Tested

Figures 3 through 6 present the results of experiments with strains L-P55, G_1, GX_3, and X_1 in various media. From the growth curves (all living cell counts) plotted on log scale, a number of simple conclusions can be drawn.

1. Glucose supported excellent growth for all strains concerned. After six to eight days of incubation without any medium change, populations increased to 10 or 12 times the original inoculum size.

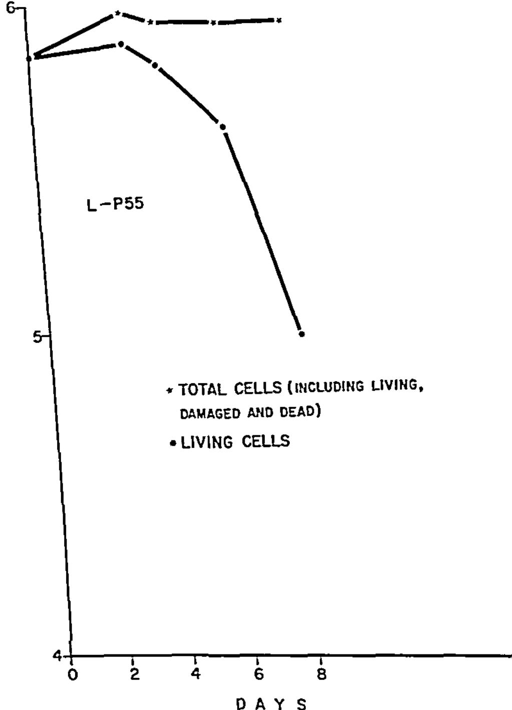


Figure 2 Strain L-P55 grown in medium without carbohydrates. Log scale showing changes in cell number of same population with respect to total counts and living cell counts only. Note that when dead cells are counted with living cells the population shows little drop in cell number.

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Figures 3 through 6 present the results of experiments with strains L-P55, G_1, GX_3, and X_1 in various media. From the growth curves (all living cell counts) plotted on log scale, a number of simple conclusions can be drawn.

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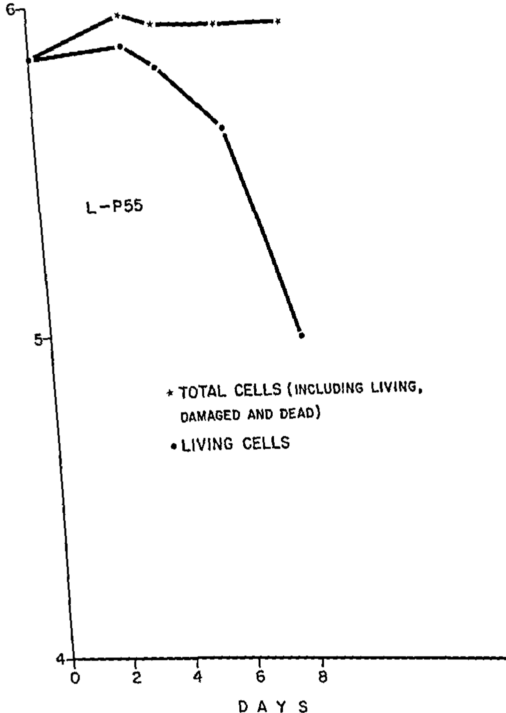


Figure 2 Strain L-P55 grown in medium without carbohydrates. Log scale showing changes in cell number of same population with respect to total counts and living cell counts only Note that when dead cells are counted with living cells the population shows little drop in cell number

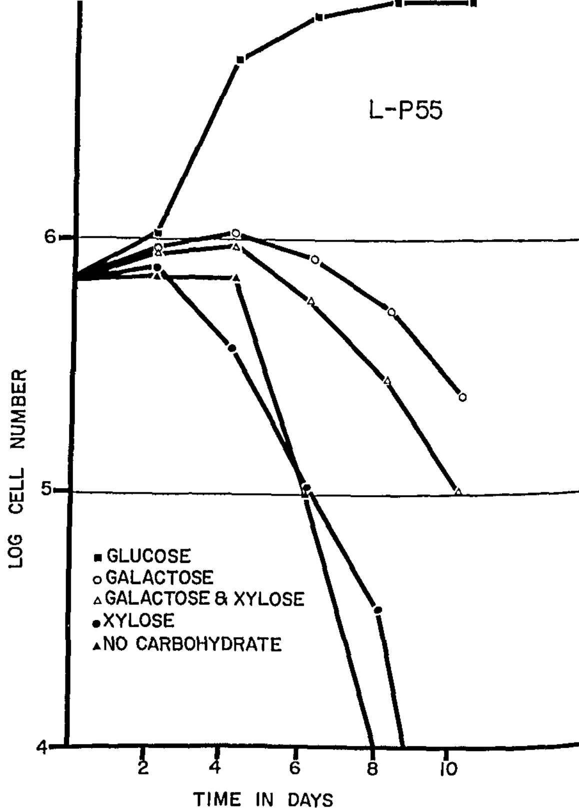


Figure 3 Growth curves (in log scale) of L-P55 in media containing glucose, galactose, xylose, galactose-xylose, and no carbohydrates.

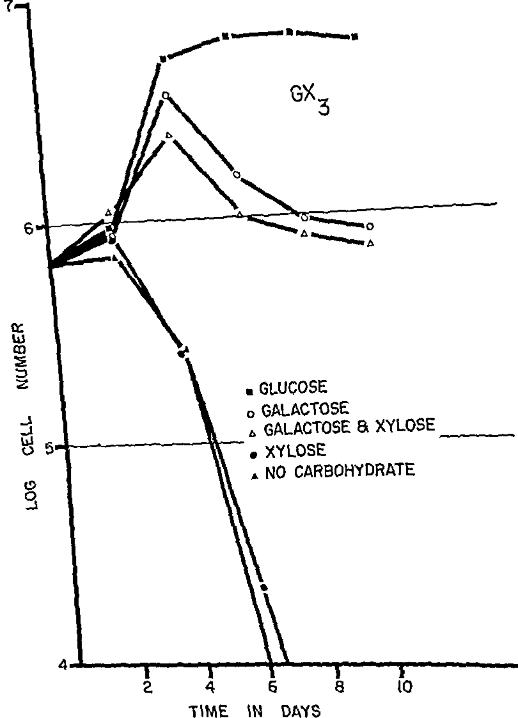


Figure 4 Growth curves (in log scale) of strain GX_3 in media containing glucose, galactose, xylose, galactose-xylose, and no carbohydrates.

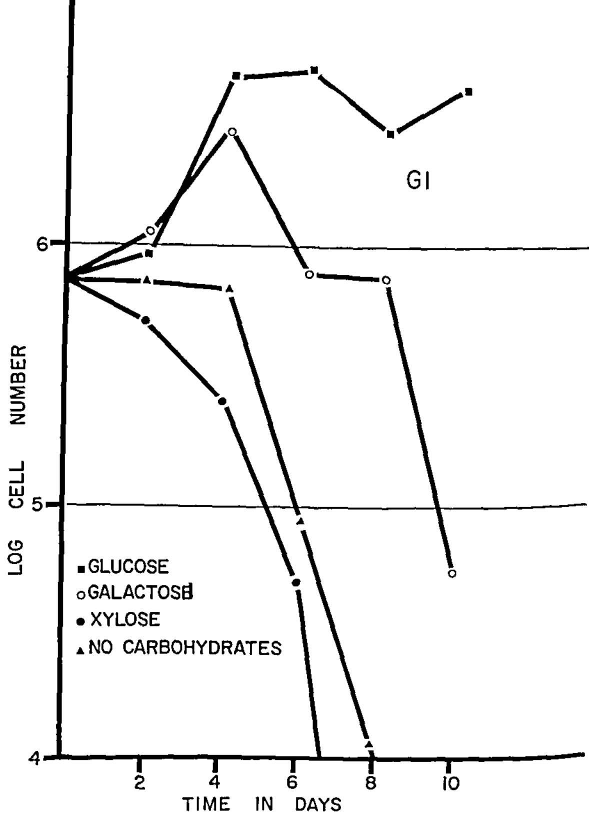


Figure 5 Growth curves (in log scale) of strain G_1 in media containing glucose, galactose, xylose, and no carbohydrates

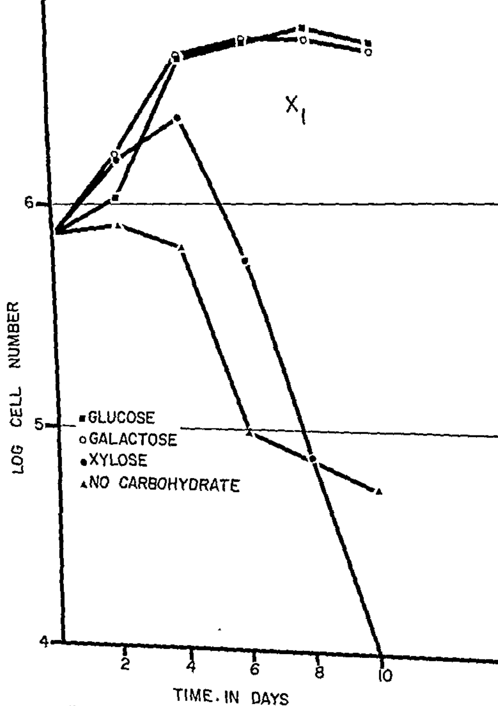


Figure 6 Growth curves (in log scale) of strain X_1 in media containing glucose, galactose, xylose, and no carbohydrates.

2. The GX strain was no more than a galactose strain. The response of the GX cells to various media was similar to that of strain G_1. In other words, the cells selected from xylose-galactose medium actually did not use xylose at all.

3. None of the strains could grow in medium without carbohydrates. The populations in carbohydrate-free medium maintained the original level or dropped slightly during the first four days of incubation, but decreased in size at an exponential rate after this period.

4. All strains except X_1 responded to xylose medium in a manner similar to the response to medium containing no carbohydrate, indicating that xylose was not utilized by these cells.

5. Cells of the parental strain showed a slight increase (near doubling) of population in galactose medium during the first four days. After this period the cell number began to drop. However, the population decrease was less rapid than that found with L-P55 cells in xylose medium or medium without carbohydrates.

6. Galactose strains (G_1, GX_3) responded to the galactose medium better than the parental line. The population might attain four times its initial size. However, it never reached the height that it did in glucose medium, and the populations soon decreased.

7. The xylose strain responded to xylose medium in an analogous fashion to the galactose strains in galactose medium.

8. The xylose strain, which had never had any contact with galactose prior to the time of the growth experiments, responded to galactose medium as well as glucose. This was the only strain tested that grew extremely well in galactose medium.

Supplementary Experiments

The most significant phenomenon observed from the preceding experiments was that the galactose strains in galactose medium and the xylose strain in xylose medium grew only during the first few days after seeding, after which the growth ability seemed to stop and the population size declined. The same number of G_1 cells grown in the same amount of medium containing glucose attained in six days two or three times as many cells as could be produced in galactose medium. Increasing the initial inoculum size did not prevent the loss of cell number, nor did the addition of galactose to the original medium after the initial period of growth exert a beneficial effect on the cultures, suggesting that the decrease in population size was not due to exhaustion of carbohydrates in the medium. Additions of

TABLE 1 Mean Values and Standard Deviations of Chromosomal Constitution of the Parental and the Metabolic Lines of L-P55

Cell Strain	Total Chromosome Number	Number of Metacentrics	Number of Subtelocentrics	Number of D Chromosomes
L-P55 (1957)	67 88 ± 1 81	12 94 ± 1 39	3 78 ± 0 86	1 20 ± 0 61
L-P55 (1958)	67 26 ± 1 91	13 30 ± 1 64	3 18 ± 1.05	0 86 ± 0.70
GX-2	67.56 ± 0 88	11 82 ± 0 98	2 08 ± 0 44	1 84 ± 0 37
GX-3	64 46 ± 2 35	13 56 ± 1.69	2 66 ± 0 69	1.20 ± 0 61
G-1	66 26 ± 1 97	13 02 ± 1.83	2 20 ± 0 75	1 66 ± 0 69
G-3	63 94 ± 2 80	13 90 ± 2.14	2.30 ± 0.70	1.42 ± 0 59
X-1	65 32 ± 1.17	14 80 ± 1 23	2 94 ± 0 45	1 00 ± 0 00
X-3	64 84 ± 2 31	14 84 ± 1.90	2 76 ± 0 71	1.16 ± 0.55

glutamine or pyruvate likewise did not produce a favorable effect though the latter has been noted by Eagle, Barban, Levy, and Schulze (1958) to be a stimulator of galactose utilization.

Chromosomes

The chromosomal constitution of the parental strain, L-P55, has been described in detail by Hsu and Klatt (1958). The strain was totally heteroploid with 68± chromosomes. There were approximately 13 metacentric and three or four subtelocentric chromosomes per cell. Most cells also contained a long subtelocentric element which had a secondary constriction. This chromosome, designated by Hsu (in press) as D chromosome, was an excellent marker.

Two samples were taken from L-P55 for cytological analysis, one in the spring of 1957 and the other in the fall of 1958. It is interesting to note from Table 1 that within the limits of sampling error, the two samples were similar except in the frequency of the D chromosome, which showed a significant decrease in the 1958 sample. In both populations the standard deviation for each class was relatively high, an indication of a heterogeneity of the populations.

The results of analysis of chromosome constitution of the sugar strains, two galactose, two galactose-xylose, and two xylose, are also presented in Table 1. With one exception, strain GX_2, in which the mean total chromosome number was similar to that of the parental line, all strains showed a drop of the total chromosome number. In the parental strain, the lowest number recorded was 63 in both samples, and cells with 65 chromosomes or lower occupied less than 10 per cent of the populations. In most sugar strains, on the other hand, cells with 65 chromosomes or lower represented the majority of the populations. The lowest limit of the chromosome number was also extended to 59. Figure 7 presents four complete photographic idiograms taken at random from cells in strain X_1. In this strain, 65 and 66 chromosomes were the most popular numbers; and each had a single D chromosome.

The number of metacentric chromosomes per cell in the sugar lines—again with the exception of strain GX_2, which contained one fewer than the parental strain—showed a tendency of increase, especially the xylose strains. There was a decrease of the number of subtelocentrics in all the sugar lines. The value of the D chromosome

Figure 7 Idiograms of four cells from strain X_1. Colchicine-hypotonic-squash Phase photomicrography.

varied from exactly one per cell in strain X_1 to almost two (1.84) in strain GX_2.

It should be mentioned that strains GX_2 and X_1 showed the smallest variability in all categories, as indicated by their low standard deviation values. In other strains, the standard deviations were either similar to, or higher than, those of the parental populations.

Discussion

There are several discrepancies in the results of sugar utilization by strain L cells between our data and those of Eagle, Barban, Levy, and Schulze (1958). For instance, strain L-P55 did not grow in xylose medium, but Eagle's L cells showed some growth response to xylose. Strain L-P55 responded to galactose medium only weakly; whereas Eagle's L cells grew in galactose medium moderately well. According to Eagle *et al.*, the addition of pyruvate to galactose medium increased growth response of *in vitro* cells, including L strain; but in our experiments, pyruvate failed to elicit any favorable growth response from L-P55. In view of the fact that we were able to establish metabolic variants from L-P55 and that polymorphism existed among sublines of strain L (Hsu, in press), it is highly possible that there were differences in metabolic patterns between these two sublines in question. Indeed, it would be misleading to describe any property of a certain subline by using its general designation, say, strain L. It is probably also important to bear in mind that even the same line carried in the same laboratory with apparently no deviation in routine culture handling cannot be characterized by results obtained during one period of time.

Actually, in comparing our data with those of Eagle *et al.*, some technical differences should also be discussed. First, in growth experiments performed in this laboratory, no medium change was made throughout the ten-day period; whereas Eagle *et al.* replenished media in the cultures every day. With frequent medium change the growth-promoting ability of a special substance can be obscured by other components of the medium which can serve partially as substitutes for the compound in question. Second, in determining growth responses, we used living cell count as our criterion; whereas Eagle *et al.* used amount of total protein as their index for growth. It can easily be seen from Figure 1 that the number of living cells dropped drastically in populations without carbohydrates; yet the total cell counts remained at approximately the initial level. Dead cells and

damaged cells definitely would contribute some protein to the samples taken, thus affecting the final estimation.

Although the present study shows that selection of metabolic variants can be made from mammalian cells, we have as yet to prove that the X_1 cells actively obtain energy from xylose for growth, because we still lack data on intermediate products of xylose utilization and enzymatic analyses. There exists a possibility that the selection was actually carried for some unknown or unsuspected factor or factors while xylose remained inert. Nevertheless, the principle that variants with inheritable metabolic differences can be obtained is unaltered, whatever the true cause may be.

For the sake of convenience, let us at the present time assume that our X_1 and X_2 lines are true xylose utilizers. The chromosome spectrum of X_1 (studied 10 months after its initiation) was much narrower than that of X_2 (studied 3 months after its initiation), although both showed a common modality of 65. It may be postulated that either X_1 arose from a single cell while X_2 derived its origin from a number of cells, or that the X_1 population originally resembled that of X_2 but repeated selection eliminated a number of weaker genomes. As far as growth responses to xylose medium are concerned, X_2 grew better than X_1, according to casual observation. It may be speculated that the original X_1 did utilize xylose more efficiently but that those genomes might have been selected against by our culturing methods, such as selecting cells adhering to the glass surface or those able to withstand adverse pH conditions, etc. In carrying a strain with some special metabolic ability, there is a possibility of selecting either more efficient or less efficient genotypes.

It is difficult with our present material to conclude whether any correlation exists between a certain karotype and a certain metabolic trait. Attempts are being made to establish more xylose lines from L-P55 for extensive cytological and biochemical comparisons. Cytological follow-up on the existing lines may also shed some light on the relations between xylose metabolism and karyotype. Perhaps by using cell lines derived from Chinese hamsters, where almost every chromosome is distinguishable, more precise information can be obtained.

Notes on Changes of Strain L-P55

In our laboratory, strain L-P55 has been maintained in two entirely different stocks since 1957, by different workers and in medium

from different containers. The second stock was carried merely as a reserve in only two culture vessels at a time, and no experiments were made with these cells. The purpose for doing so was to avoid losing the strain in case of laboratory disasters. The main stock was heavily propagated for experiments reported in this study. Soon after the completion of the growth curve assays, however, a yeast contamination wiped out all the cultures of the stock in use. We were obligated to propagate the spare stock for continuation of our investigations. The first surprising phenomenon noticed was the fact that the cells in the spare stock were able to utilize galactose much more efficiently than the original stock, although their response to xylose did not alter significantly. Chromosomal analysis made by Dr. G. K. Manna showed that the population of the spare stock was also quite different from the original one. The mean chromosome number per cell was 64.16, with 13.96 metacentrics, 2.04 subtelocentrics, and 0.64 D chromosomes. Besides, there was an unusually large metacentric element hitherto unrecorded in L cells. Generally this chromosome appeared in cells where the D chromosome was absent.

This unexpected and perhaps rather unpleasant change in the strain shows that spontaneous changes in cytological as well as metabolic features of a cell population may occur without special efforts at selection. This probably could account for a number of discrepancies found in the literature where workers study the same phenomenon with the same strain of cells.

Summary

Strain L-P55, a subline of clone 929 of strain L, was treated with media containing galactose, xylose, galactose-xylose combination, lactose, or sucrose. Progressive death of the cultures began to occur four to five days following the treatment. In all cases except xylose, scattered survivors were noted throughout the containers. These cells were kept in their respective media and eventually repopulated their containers to give rise to the special sugar strains. In xylose, however, practically all the cells died. Out of approximately 100 million cells treated with xylose medium, only three colonies, occurring in separate containers, developed into xylose strains.

Growth curves were constructed for the parental line, a xylose line, and a galactose line In glucose medium luxuriant growth was recorded in all the lines, and control medium lacking carbohydrates supported no growth. Galactose supported growth for the galactose strain, but under the cultural conditions of growth curve experiments

the maximum population density attained was about 4 times the inoculum level. This maximum was followed by a rapid decline in the population. Neither use of an inoculum of twice the usual cell numbers nor periodic additions of the carbohydrates had a commensurate effect on the population decline. Periodic addition of a vitamin mixture and/or amino acid mixture produced a maximum population density of six to eight times the inoculum, and provided a stable population of approximately four times the inoculum level. For the parent line, galactose supported only a very limited growth; but the cultures deteriorated at a slower rate than that observed with carbohydrate-free medium. The xylose strain X_1 could obtain from galactose the same quantity of cell growth as from glucose. Medium containing xylose did not support growth of any line except the xylose strain

Cytologically, strain L-P55 was characterized by having 68± chromosomes with 13± metacentrics. In the sugar strains, no definite chromosome patterns were found to correlate with their metabolic abilities However, many sugar strains possessed a lower chromosome number but a higher number of metacentrics.

These experiments suggest that hereditary metabolic variants can be isolated from cultures containing populations of mammalian cells The variants, or, loosely speaking, "mutants," are useful tools for studies in various fields.

ACKNOWLEDGMENTS

Supported in part by Grants DRG-269D from the Damon Runyon Memorial Fund for Cancer Research and P-133A from the American Cancer Society

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Gene Action

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An understanding of gene action is essential to a rational understanding of growth, be it normal or abnormal growth. At present, however, one cannot describe gene action. Rather, one is limited to a discussion of the current hypotheses, for while we all enjoy a good deal of optimism, we do not yet completely understand the function of genetic material, and it is certain that the years ahead will provide many surprises in this field in terms of our present knowledge and preconceptions

In the course of the past 20 years geneticists, biochemists, and physicists have made remarkable strides in advancing from a description of gene action in morphological terms to its description in enzymatic terms. In fact, it will shortly be possible to discuss the action of a gene in terms of its determination of specific amino acid sequences. An understanding of the action of genetic material in terms of enzyme specificity is necessary for an understanding of growth. However, is this action the sole characteristic of the gene that concerns the student of genetics and cancer?

Our knowledge of the characteristics of genetic material has been tremendously enhanced in recent years. We know that meiosis is not a prerequisite for genetic recombination; somatic recombination does occur, as does genetic recombination in nonmeiotic organisms. We also know that determination of enzyme specificity is not necessarily the only function of genetic material It may still have additional

functions of which we are only dimly aware at the present time. These functions may be complex and all of these complexities undoubtedly bear on the problems of genetics and cancer. First, let us consider the evidence which leads to the belief that one action of genetic material is the specification of enzyme structure.

Genes and Biochemical Reactions

If one looks over the history of genetic thought he is impressed by the fact that geneticists have always held a deep conviction that genetic material must ultimately regulate the biochemical activities that a cell performs, for the phenotype must inevitably reflect the biochemical activities carried on by the organism. The experimental realization of this conviction, however, required time. The experimental demonstration of the genetic control of biochemical activity was particularly clearly demonstrated in the now classic experiments of Beadle and Tatum, who used the fungus *Neurospora crassa* (Beadle and Tatum, 1941), to show conclusively that as a consequence of genetic change the organism loses the ability to synthesize essential nutrients. Detailed biochemical and genetic study of such nutritional mutants showed that the specific biosynthetic reactions enjoyed by a cell are controlled by specific genes, and that the ability to perform a specific biochemical reaction is apparently controlled by a specific gene (see reviews in Beadle, 1945; Bonner, 1946). Biochemical reactions require enzyme catalysis, and, inasmuch as genetic change appears to result in the loss of ability to carry out specific biochemical reactions, it seems reasonable to assume that this loss of biochemical activity must reflect an alteration in enzyme-forming ability, either in a quantitative or a qualitative sense. The genetic control of enzyme formation has been demonstrated in many instances (see review in Beadle, 1957). In fact, one can now state with assurance that one function of genetic material is the control of enzyme formation. This is in reality the starting point of the present discussion, since a point of major interest is the nature of the relationship between genes and the process of enzyme formation.

Genes and Enzymes

During the past several years the genetic control of a number of enzymes has been investigated (see review in Beadle, 1957). Our own investigations have been largely concerned with the genetic control of one enzyme, tryptophan synthetase, and it is the genetics of this enzyme that will be primarily discussed. The data which will be

ANTHRANILIC ACID

INDOLE-GLYCEROL-PHOSPHATE + SERINE → TRYPTOPHAN

+ SERINE

INDOLE

TRYPTOPHAN SYNTHETASE

Figure 1a Formation of tryptophan in *N. crassa*.

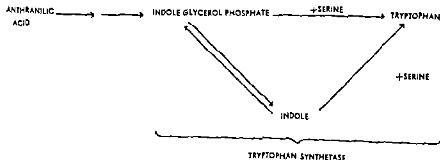


Figure 1b Formation of tryptophan in *E. coli*

cited have been obtained in part at Yale, and in part by Dr. Charles Yanofsky of Stanford University and Dr. S. R. Suskind of the Johns Hopkins University. Tryptophan in *N. crassa* and *Escherichia coli* is normally formed from anthranilic acid via the intermediate compound indole-3-glycerol-phosphate (Yanofsky, 1956, 1957; Yanofsky and Rachmeler, 1958). This latter compound is converted to tryptophan by an exchange reaction with serine which is catalyzed by the enzyme tryptophan synthetase. Tryptophan synthetase also catalyzes two other reactions, the conversion of indole-3-glycerol-phosphate to indole, and the condensation of indole and serine to tryptophan. That all three of these reactions are catalyzed by the same enzyme has been thoroughly demonstrated (Crawford and Yanofsky, 1958; Yanofsky and Rachmeler, 1958; DeMoss, Imai, and Bonner, 1958; Suskind and Jordan, in press). It is possible to isolate mutants lacking tryptophan synthetase activity. Such mutants are characterized by a growth requirement for tryptophan which cannot be replaced by indole. During the past several years a number of strains have been independently isolated, all of which require tryptophan and cannot utilize indole for growth, and all of which lack detectable amounts of the enzyme tryptophan synthetase.

With such a group of mutants one can perform a number of experiments. For instance, one can determine whether all of the genetic information relating to the formation of this one enzyme is contained within a single region of the genetic material of *N. crassa* or is present in a number of different regions. This question can be answered by crossing the mutants with each other and studying linkage relationships. Experiments of this sort permit us to say with a good deal of assurance that all of the major information relating to the formation of this enzyme is contained within a restricted region of a chromosome of this organism (Yanofsky and Bonner, 1955; Lacy, 1959). This restricted region of one chromosome has been called the td locus, and mutants having mutations in this region are called td mutants (Yanofsky and Bonner, 1955).

The genetic complexity of this region is of interest. Does this region consist of a single site which undergoes change and so gives rise to a uniform class of mutants, or are there many sites which when altered can effect the formation of this enzyme, each in a unique way? These questions can be answered experimentally by determining whether the mutant strains which lack the ability to form tryptophan synthetase are identical or not. It can be clearly shown that the region must be mutationally complex. In fact, one is left with the impression

that if a sufficient number of criteria were used, few identical mutants would be found. For instance, only one of the hundred mutants that have been isolated is temperature sensitive (Yanofsky and Bonner, 1955). Only two of the strains accumulate the compound indole (DeMoss, Imai, and Bonner, 1958). Still additional methods of comparison can be used. The mutants can be tested for the presence of a material which is serologically related to the parental enzyme. When extracts of mutant strains are examined for the presence of such material one finds that certain mutant strains do form a protein which will react with and remove tryptophan synthetase antibodies.[1] This is not true of all of the mutants Other mutational events, which also result in loss of tryptophan synthetase activity, lead to the complete loss of ability to form a protein which is serologically related to the parental tryptophan synthetase (Suskind, Yanofsky, and Bonner, 1955).

One additional type of difference might also be mentioned. It deals with the effect of mutations at still other regions in the chromosomes Certain td mutants regain the ability to form tryptophan synthetase as a consequence of a mutational event at a second and distinct locus (Yanofsky, 1952, Yanofsky and Bonner, 1955). Such mutations are

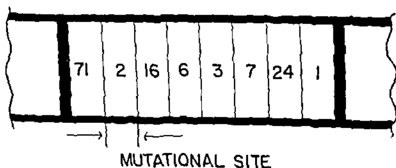


Figure 2 Region of the chromosome controlling the formation of tryptophan synthetase

referred to as suppressor mutations. An interesting characteristic of the suppressors is that they are allele specific, and this specificity supports the view that these independent mutations of the td locus differ (Yanofsky and Bonner, 1955). Thus, by comparing these strains in

[1] Material which will react with and remove tryptophan synthetase antibodies, and which is inactive in catalyzing the reaction: indole + serine → tryptophan, is referred to as CRM (cross-reacting material).

a number of different ways, one is led to the conclusion that the region of the chromosome which controls the formation of tryptophan synthetase is a complex region with many potential mutational sites. What are these mutational sites? Do they represent elements of the gene which can undergo recombination? Must they be present in a specified order to permit the formation of enzyme having a specific catalytic characteristic?

The problem of the discreteness of these mutational elements is difficult to answer. Interallelic crosses yield rare prototrophic progeny (Lacy, 1959). However, in order to answer the question of the discreteness of these elements one needs to know whether prototrophic progeny formed in interallelic crosses arise by a reciprocal or crossover type of recombinational event or by a nonreciprocal or copy-choice type of recombinational event. Interallelic crosses of the td mutants do not appear to give the results predicted of reciprocal recombination, though this point lacks good documentation. In many respects the results from the td locus are similar to those obtained with the more extensively investigated Q locus (St. Lawrence, 1956), which indicates that prototrophic progeny from interallelic crosses do not arise by reciprocal recombination. Thus, the recombinations arising from these interallelic crosses differ from those arising from crosses involving mutants blocked in the formation of different enzymes. The latter always show reciprocal recombination (Bonner, 1956). The reason for the difference in behavior of the two types of recombinational units is not known, but it might reflect the inability of DNA to undergo the breakage fusion required of a crossover event.

Complementation

From the data reviewed, one can arrive at certain definite conclusions concerning the nature of the genetic region controlling the formation of tryptophan synthetase. The genetic information controlling the formation of this one enzyme appears to reside in a restricted region of a single chromosome of the organism. This region appears to be complex in terms of number of mutational sites and number of nonreciprocal recombination units, but indivisible in terms of reciprocal recombination. It therefore becomes of interest to determine whether the formation of tryptophan synthetase requires that all of the elements which can be distinguished as mutational sites be present not only in a given configuration but in a given sequence as well. In *Neurospora crassa* it is not possible to answer this question directly by means of the diploid cis/trans test (Lewis, 1951; Ponte-

corvo, 1956). Neurospora has a transient diploid phase and it is therefore impossible to study the enzymatic characteristics of the heterozygote. Neurospora will, however, form stable heterocaryons; so one can see if enzyme formation will occur when all of the mutational elements required for the formation of tryptophan synthetase in the parental strain are present in a common cytoplasm but distributed among different nuclei (for discussions of heterocaryons see Beadle and Coonradt, 1944; and Garnjobst, 1953).

Experimentally, one can determine whether or not heterocaryons formed between different td mutants are capable of growing in the absence of exogenous tryptophan, and, if they do grow, whether or not they are capable of forming an active tryptophan synthetase. This problem was first examined a number of years ago, at which time it was concluded that heterocaryons formed between different td mutants lack the ability to form tryptophan synthetase. However, after it had been shown by Giles, Partridge, and Nelson (1957) that certain allelic pantothenic-requiring mutants formed adenine-independent heterocaryons, the td mutants were re-examined. It has been found (Lacy and Bonner, 1958, Lacy, 1959) that certain td mutants form heterocaryons which will grow in the absence of tryptophan and which will form an active tryptophan synthetase. Not all of the td mutants show this phenomenon of complementation. In fact, one can recognize certain characteristics which appear to govern the ability of td mutants to complement (Lacy and Bonner, 1958). All of the strains which show complementation still retain the ability to form a protein serologically related to the parental enzyme. Additionally, it appears that the two complementing strains must differ in terms of their functional alteration in tryptophan synthetase formation. For instance, two strains both of which are suppressed by a common suppressor will not complement each other, but will complement with the two strains which accumulate *indole*. In turn the two indole-accumulating strains will not complement each other. As shown in Figure 3, by means of overlapping bar graphs one can draw a complementation map of the td region. Such a map can be constructed by putting the strains with identical complementation patterns together in groups, and by overlap indicating strains which may not complement with each other but whose complementation patterns differ. Such a map suggests that the td region consists of at least three major complementation areas. A similar and more detailed map of the adenylosuccinase locus had been prepared by Woodward, Partridge, and Giles (1958). One question of obvious interest is whether

or not the relative positions of alleles in such a map are about the same as those found in a recombination map. This question has not been clearly answered in the case of the td mutants, though such a correlation does appear to exist (Lacy, 1959). Such a correlation, however, has been shown by Case and Giles (1958) in studies of a pantothenic locus. Perhaps the point of major interest for the moment is that although the td region, judged in terms of mutation, is found to consist of many sites, judged in terms of complementation it appears to be a region of only a few sites. Thus, each complementation group appears to contain many mutational sites. What are these

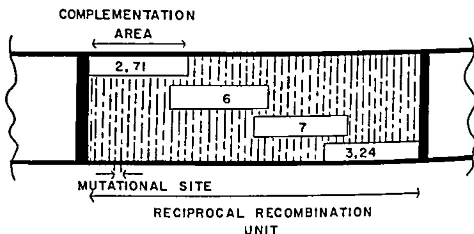


Figure 3 Genetic area controlling tryptophan synthetase formation

complementation units? Do they represent discrete functional areas of the td locus? At present we cannot answer these questions. The explanation for complementation probably ultimately hinges on an understanding of the changes in enzyme formation that are associated with gene change. Before complementation can be discussed further, therefore, the characteristics of mutant enzymes must be considered.

The Mutant Enzyme

The experimental data available at the present time strongly suggest that mutation results in the formation of qualitatively altered proteins. This can be documented in the case of tryptophan synthetase in a number of ways. As was discussed earlier, one td mutant is

temperature sensitive. This strain requires tryptophan if grown at 25°C, but is tryptophan independent if grown at 35°C. This strain may be suppressed, and will then grow in the absence of tryptophan at 25°C. When grown at 25°C it characteristically forms a protein which is serologically similar to tryptophan synthetase. Suskind and Kurek (1957) have observed that by dialysis this protein can be activated to form a catalytically active tryptophan synthetase. The enzyme obtained by activation of the cross-reacting material differs from the enzyme of the parental strain in its greater sensitivity to zinc inactivation. Since the enzyme prepared by activation of the cross-reacting material appears to be an altered enzyme, it is of interest to determine the characteristics of the enzyme formed by this mutant under a number of different conditions. Suskind and Kurek (1957) have carried out this comparison. Interestingly enough, regardless of how this strain is persuaded to form tryptophan synthetase, the

TABLE 1. Comparison of Characteristics of Enzyme Obtained by Activation of Cross-reacting Material under Different Conditions

GENOTYPE	ZINC SENSITIVITY
td_{24} —25°C→ CRM —dialysis→ T'Sase	+
td_{24} —35° C→ T'Sase	+
td_{24} —25° C or 35° C→ T'Sase	+
td(wild type) —25° C or 35° C→ T'Sase	-

formed enzyme is always sensitive to zinc inactivation. Thus the presence of the td_{24} gene always results in the formation of an enzyme which differs from the tryptophan synthetase formed by the parental strain, and it is clear that the characteristics of the enzyme are determined by the nature of the td locus.

The fact that the td_{24} gene gives rise to a zinc-sensitive enzyme clears up questions relating to the temperature sensitivity and sup-

pressibility of the strain. The temperature-sensitive characteristics of the strain could be explained by the hypothesis that the uptake or synthesis of some normal component which now acts as an inhibitor of the td_{24} enzyme, such as zinc, is impaired at 34°C. As regards suppressibility, the data of Suskind and Kurek (1957) clearly show that the suppressor gene does not contribute to tryptophan synthetase formation, as the enzyme of the suppressed strain is similar to the enzyme obtained by activation. The suppressor gene in this case appears to act indirectly and could act by regulating the uptake or synthesis of a normal component that acts as an inhibitor of the mutant enzyme.

Study of the strains which accumulate indole also leads to the similar conclusion that mutation results in the formation of altered enzymes. Tryptophan synthetase normally catalyzes a reaction involving an exchange between serine and indole-3-glycerol-phosphate (Yanofsky and Rachmeler, 1958). The same enzyme, however, catalyzes two additional reactions, one involving the conversion of indole-3-glycerol-phosphate to indole, and another involving the condensation of indole and serine to give tryptophan. The strains which accumulate indole form a protein serologically related to the tryptophan synthetase of the parental strain. This protein lacks the tryptophan-forming reactions, but it retains the indole-forming reaction (DeMoss, Imai, and Bonner, 1958). This finding indicates that mutation has led to formation of an altered enzyme. In this instance the alteration appears to interfere with a very restricted portion of the enzyme molecule, specifically, the combining site for serine.

A last point bearing on mutation and the formation of qualitatively altered enzymes is the fact that the majority of ultraviolet-induced mutants retain the ability to form a serologically related protein. From the preceding discussion, it seems likely that these serologically related proteins are altered tryptophan synthetase, but the precise nature of the alterations has not yet been elucidated. As certain of these mutants are known to represent mutations at different sites and as cross-reacting material may well represent altered enzyme, it appears that mutation in the td region commonly leads to the formation of altered enzyme.

All of these data stress the correlation between genetic change and qualitative enzyme alteration. In view of Ingram's (1957) work on the single amino acid differences in human hemoglobins, the obvious prediction that one would make at present is the prediction that mu-

tations within the td region lead to slight changes in the amino acid sequence of the tryptophan synthetase molecule. Substitution of one amino acid for another, or deletion of one amino acid, could lead to the formation of a catalytically inactive enzyme. The substitution of one amino acid by another could lead to the formation of a protein whose physical characteristics might not permit it to survive within the cytoplasm. It might lead to the formation of an enzyme which could be inactivated by some normal cellular component. Or it might lead to the formation of a protein altered in such a way that it could not combine with the substrates.

This line of reasoning leads to the hypothesis that the td region is one which determines the amino acid sequence of tryptophan synthetase, and that the smallest site measured by mutation is one governing the specification of a single amino acid. This is not a novel idea. In fact, quite the contrary, as it has been this general line of thought that most geneticists have followed in recent years. To follow this same line of reasoning a bit further, since all available data point to DNA as the genetic material, each mutational site could be one or a few nucleotide pairs. The problem of how DNA controls the amino acid sequence still awaits elucidation. Zalokar (1958) has recently shown that in Neurospora RNA is formed in the nucleus and migrates to the microsomes, while protein is formed both in the nucleus and in the microsomes. This reinforces the thought that gene action, in governing enzyme structure, involves the triumvirate of DNA, RNA, and protein, but an understanding of the actual role of each must await clarification of protein biosynthesis.

Having arrived at the conclusion that mutations at the td locus lead to the formation of altered enzymes, let us reconsider the phenomenon of complementation. The fact that complementation occurs could suggest that two altered enzymes might interact in the cytoplasm and so reconstitute enzymatic activity. The very fact that enzymatic activity may be masked by inhibitors, as has been shown in the case of the temperature-sensitive mutant, means that complementation, in part, could represent cytoplasmic activation. While it is likely that such a mechanism will be found to account for a number of complementing pairs, it is difficult to account for all cases of complementation in this way.

During the past year three extraordinarily interesting observations which deal with the structural nature of enzymes have appeared. One observation is that of Richards (1958), dealing with the struc-

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might be bound together by covalent linkages but, since catalytic activity may not require their being so bound, complementation could further involve the association of these components by electrostatic binding. Such speculations are of great interest at the present time but require experimental verification Crawford and Yanofsky (1958) have shown that extracts of mutant strains of *E. coli*, one of which is defective in the A protein and the other defective in the B protein, can form tryptophan synthetase activity by dissociation and hybridization. They have further shown that the genetic areas controlling the formation of the A and B protein are closely linked. It has not been possible to dissociate the tryptophan synthetase of Neurospora into independent molecules. However, it might be possible to break certain covalent bonds of this enzyme and so free the independent protein molecules that constitute the whole enzyme. Thus, whether a number of discrete polypeptide molecules constitute the tryptophan synthetase of *N. crassa* has still to be experimentally determined, and the correlation between the complementation map and possible independent polypeptide molecules remains an intriguing possibility.

The fact that enzyme molecules may consist of a number of independent polypeptide molecules must be borne in mind in considering the problem of genes and enzymes Such considerations could have a profound influence on the problem of the genetic control of enzyme formation and even on the problem of gene action itself. It is clearly possible that if this were true a given molecule might serve in more than one enzyme complex. Evidence supporting this type of relationship has not yet been obtained. However, the experiments of Atwood and Mukai (1953) show that the mutant strains which have so far been investigated genetically represent but a small fraction of the total mutants, and it may be that our methods of selection of mutant types have precluded the isolation of strains in which a relationship of this sort would be evident. In conclusion, one can only emphasize the fact that the structural nature of enzymes themselves must be carefully considered when discussing the problem of gene action, for it is possible that what we now call the td locus is in reality a number of closely linked loci, each one of which controls the formation of a separate polypeptide molecule.

We have discussed the relation of genes to enzymes and the alterations that may arise from mutation What is their relation to cancer? Mutation and altered enzymes clearly could be involved in the origin of cancer. At the present time, however, we are not in a position to make any clear suggestion as to what type of alteration might result

ture and activity of ribonuclease. Richards treated ribonuclease with the proteolytic enzyme subtilisin, and found that following this treatment, two polypeptides, one large and one small, can be isolated. Neither of these two polypeptides by itself has enzymatic activity. However, the mixing of the two at an appropriate pH leads to the restoration of ribonuclease activity without the concomitant formation of the original intact polypeptide chain. These observations suggest that while ribonuclease normally consists of one polypeptide molecule, it can be split into two polypeptide molecules which can associate in an effective manner by electrostatic bonding. The tryptophan synthetase of *E. coli* has been studied in detail by Crawford and Yanofsky (1958), who made the exciting discovery that fractionation of *E. coli* tryptophan synthetase results in the isolation of two protein fractions neither one of which has enzymatic activity, judged in terms of any of the three reactions catalyzed by this enzyme. However, full activity is restored when these two proteins are mixed. *E. coli* tryptophan synthetase, therefore, appears to consist of at least two protein molecules which are normally held together by electrostatic forces. The third observation of interest is the studies of Singer and Itano (in press) on hemoglobin. Hemoglobin consists of four polypeptide chains of two types. Under appropriate conditions hemoglobin splits into two subunits containing two identical chains. In mixing experiments with *hemoglobin A* and *hemoglobin S*, Singer and Itano (in press) have shown that asymmetric dissociation and hybridization can occur. All of these observations point to the fact that enzymes may normally consist of a number of discrete polypeptide molecules which may or may not be held together by covalent bonds. Perhaps of greater interest to the present discussion, however, is the suggestion that the catalytic activity of an enzyme need not be dependent on having all of the polypeptide components of an enzyme bound together as a single molecule. Such evidence obviously suggests that the genetic areas delineated in a complementation map could represent regions required for the formation of specific polypeptide molecules. Thus complementation could reflect the fact that Region A is required for the formation of a polypeptide molecule A, Region B for the formation of a polypeptide molecule B, and Region C for the formation of a polypeptide molecule C. Mutant strains having an alteration in Region A might then be expected to complement with mutant strains having a defect in either Region B or C, since the two strains together would be able to form the normal A protein as well as the normal B and normal C protein. Normally these polypeptides

might be bound together by covalent linkages but, since catalytic activity may not require their being so bound, complementation could further involve the association of these components by electrostatic binding. Such speculations are of great interest at the present time but require experimental verification Crawford and Yanofsky (1958) have shown that extracts of mutant strains of *E. coli*, one of which is defective in the A protein and the other defective in the B protein, can form tryptophan synthetase activity by dissociation and hybridization. They have further shown that the genetic areas controlling the formation of the A and B protein are closely linked. It has not been possible to dissociate the tryptophan synthetase of Neurospora into independent molecules. However, it might be possible to break certain covalent bonds of this enzyme and so free the independent protein molecules that constitute the whole enzyme. Thus, whether a number of discrete polypeptide molecules constitute the tryptophan synthetase of *N. crassa* has still to be experimentally determined, and the correlation between the complementation map and possible independent polypeptide molecules remains an intriguing possibility.

The fact that enzyme molecules may consist of a number of independent polypeptide molecules must be borne in mind in considering the problem of genes and enzymes. Such considerations could have a profound influence on the problem of the genetic control of enzyme formation and even on the problem of gene action itself. It is clearly possible that if this were true a given molecule might serve in more than one enzyme complex Evidence supporting this type of relationship has not yet been obtained. However, the experiments of Atwood and Mukai (1953) show that the mutant strains which have so far been investigated genetically represent but a small fraction of the total mutants, and it may be that our methods of selection of mutant types have precluded the isolation of strains in which a relationship of this sort would be evident. In conclusion, one can only emphasize the fact that the structural nature of enzymes themselves must be carefully considered when discussing the problem of gene action, for it is possible that what we now call the td locus is in reality a number of closely linked loci, each one of which controls the formation of a separate polypeptide molecule.

We have discussed the relation of genes to enzymes and the alterations that may arise from mutation. What is their relation to cancer? Mutation and altered enzymes clearly could be involved in the origin of cancer. At the present time, however, we are not in a position to make any clear suggestion as to what type of alteration might result

in neoplastic growth. For the purpose of the present discussion it must suffice to point out that a number of cases are now known in microorganisms and in man in which the formation of an altered enzyme is clearly associated with a mutant form of a specific genetic area. Thus in man as in microorganisms it follows that genetic material can control the qualitative characteristics of specific enzymes.

GENE ACTION AND THE QUANTITATIVE REGULATION OF ENZYME FORMATION

Is the qualitative nature of the enzyme the only aspect of enzyme formation which is genetically determined? This is a problem which geneticists have long pondered (Goldschmidt, 1937). It is of course well known that not all of the genetic information of a cell is functional at any point in development. In Neurospora certain enzymes are formed in quantity relatively late and others early in the growth of a developing hypha (Zalokar, in preparation). In fact, there is abundant evidence that enzyme formation is a regulated process but, unfortunately, there is a scarcity of evidence bearing on the mechanism of this regulation. In the past two years, quantitative aspects of enzyme formation have been widely considered. Enzyme repression and feed-back mechanisms have been proposed and documented experimentally (Vogel, 1957; Gorini and Maas, 1957; Yates and Pardee, 1957). These mechanisms have been largely discussed in terms of their operation at the enzyme-forming level, and while they unquestionably could have an ultimate genetic basis, the role of genetic material has not been seriously discussed. It is this role, however, with which we are concerned here. Does genetic material serve as a regulator of the quantitative aspects of enzyme formation? If it does, is this then an action of genetic material inseparable from the locus that determines the qualitative characteristics of the enzyme? If it is separable, is it linked to that locus, or is it unrelated? Lastly, does each enzyme have a regulator element, either built in, linked or unrelated, or can one regulator element regulate a whole biochemical pathway? These are exciting and intriguing problems, but, unfortunately, they are difficult to solve at the present time.

In Neurospora, genes can affect the rate and extent of enzyme synthesis. This phenomenon has been particularly well studied in the case of β-galactosidase (Franklin, 1954). A number of genes are known to exert a quantitative influence on the formation of β-galactosidase. The genes which have been studied, however, appear to

influence the amount of enzyme formation indirectly. For instance, one gene is known that limits the uptake of lactose, and thus the concentration of the inducing sugar is reduced in such mutants and the rate of enzyme formation is lowered. This gene appears, then, to affect primarily the permeability characteristics of the organism without directly affecting its ability to form β-galactosidase Still other genes with other indirect effects are known to affect the formation of this enzyme. Such genes do serve in a regulatory fashion, but it is not evident from the data that modifier genes, as these might be called, have as their primary function the quantitative regulation of enzyme formation. At present only fragmentary bits of evidence are available which suggest that there may be a quantitative regulatory aspect to the problem of gene action Hartmann *et al* (in press) have studied genetic control of histidine formation in Salmonella, showing that the genes controlling the various biochemical steps in histidine formation are linked together and appear to be linked in the same order as the reaction sequence. Two genes are known which appear to represent novel phenotypes, in that these two genes do not associate readily with a particular biochemical reaction or single amino acid deficiencies. Interestingly enough, these genes are at the opposite ends of the linkage group and so appear to enclose the other histidine loci which have been associated with single enzyme deficiencies. Hartmann's group has suggested that these two genes could represent the regulatory elements for histidine formation, and so one or two genetic elements might serve as the genetic regulatory elements for an entire biosynthetic pathway In studying the genetic control of tryptophan formation in *E. coli*, Eisenstein and Yanofsky found (personal communication) that when genetic material of Shigella is transferred to *E. coli* by transduction, the resulting organism forms tryptophan synthetase in excess (100 × the normal amount). It is possible that a regulator gene may control the quantitative aspects of tryptophan formation, and that this excess enzyme formation might reflect the fact that the linkage relationship of the regulator gene to the genes controlling tryptophan formation differs in *E. coli* and Shigella Giles and his colleagues (Giles, Partridge, and Nelson, 1957) have observed in a study of adenylosuccinase revertants in Neurospora that occasionally a revertant is isolated which forms more enzyme than the parental strain. A similar observation has been made in the study of revertants of td mutants. The latter observation can be variously interpreted, but it too suggests a regulator gene. The investi-

gations of McClintock (1951, 1956) on the controlling elements of corn suggest a regulatory aspect of gene action, as do the modulator genes of corn which have been described by Brink (1954). All these observations support the general view that genetic elements are involved in the quantitative as well as in the qualitative control of enzyme formation. Whether these two aspects of gene action are inseparable at a single locus or are in separable elements is not known, though present evidence perhaps favors the view of linked but separate areas. Thoughts of this sort are of particular interest to the student of cancer, because in cancer we are dealing with problems of unregulated growth. While we have scant evidence at the present time, it may well be that experimental work will soon provide direct evidence concerning the genetic nature of the regulatory mechanisms governing enzyme formation. Evidence of this sort will be obtained in many different ways. For instance, it will be of interest to find whether gene mutations resulting in unregulated enzyme formation can be found. It will also be of interest to study the action of the same genetic material in different hosts. The future may well prove kind, and it may soon be possible to transfer genes from one fungus to another, as we can do at present only in bacteria. More exciting is the possible addition of genes of a tumor cell to a normal cell. If this can be done, the problem of enzyme regulation in cancer will become a very exciting field.

In conclusion, we can say of gene action that genes specify the amino acid sequence of enzymes. How genes do this and what relationships exist among the triumvirate DNA, RNA, and protein in this action cannot be intelligently explained until we know far more about the biosynthesis of protein. For the future, perhaps, an even more important problem is the discovery of other functions, such as a quantitative regulatory function, which genetic material might fulfill; for it is in this field that we may find answers which will prove of particular profit to the understanding of cancer.

ACKNOWLEDGMENTS

The work reported in this paper has been supported in part by the Atomic Energy Commission, and in part by the American Cancer Society.

The author wishes to express his appreciation to his many colleagues whose work made this paper possible. The author also would like to thank Dr. Barbara Bachmann Beam and Dr. John A. DeMoss for their aid in the preparation of this manuscript.

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The work reported in this paper has been supported in part by the Atomic Energy Commission, and in part by the American Cancer Society.

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in the induced tumors. Response to the most potent chemical carcinogens can be demonstrated to be greatly varied by altering the genotype. Such alteration can be in respect to degree of response of a particular type of tumor and also in respect to the type of tumor induced. The list of tumors associated with viruses is increasing, but viruses, as well as the response of the host to the virus, are often under the influence of genes.

The eventual conclusion is that the occurrence of all types of tumors in all species is probably to some degree under the influence of genes. Having reached this point, however, one must realize that this all-inclusive conclusion represents only the first goal in the study of the genetics of cancer. A goal much greater than demonstrating the influence of the gene is that of physiologically linking the gene to the occurrence of the cancer. How do the genes influence the occurrence of the tumor? It is in this area of physiological genetics that the future's most important work of the genetics of cancer lies. Considerable progress has been made in the study of gene action paths leading to the occurrence of certain types of cancer; in no case have all the steps linking the primary gene action to the final appearance of the neoplasm been identified.

Gene action as used in this discussion does not refer to the primary gene action which is strictly that of the gene reproducing itself. Rather, it refers to the links in the chain of events connecting the gene to the tumor. These steps can be localized anatomically, i.e., the identification of the organs, tissues, or cells in which they occur; physiologically, i.e., the identification of the physiological pathways initiated and directed by the genes, and finally biochemically, i.e., the exact biochemical reactions and their associated genes in the physiology leading to the occurrence of the tumor.

Techniques of transplanting an organ of one genotype into a host of a different but immunologically compatible genotype have now been used extensively in determining whether the gene action resulting in a tumor in the transplanted organ is limited to that organ or is manifest through some systemic mechanism of the host. In our own laboratory pulmonary tumors of the mouse have been investigated in this way. In the original experiment, segments of lung from young adults of both the susceptible strain A and the resistant strain C57L were transplanted subcutaneously into the (A × C57L)F_1 hybrids (Heston and Dunn, 1951). Since the F_1 inherited all the dominant genes from both parent strains, it accepted the transplants from both parent strains. In earlier studies it had been estimated that these

Site of Gene Action and Carcinogenesis

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In the overall consideration of the etiology of neoplasia in all species, genes are probably the most influential of any set of factors in determining whether or not cancer occurs. In mice, through the development of the inbred strains, through segregation studies, and through linkage studies, the influence of genetic factors has been demonstrated for mammary gland tumors, hepatomas, pulmonary tumors, leukemia and other reticulum cell neoplasms, gastric tumors, papillomas of the skin, carcinomas of the skin, subcutaneous sarcomas, osteogenic sarcomas, lipomas, hemangio-endotheliomas, adenomas of the pituitary gland, tumors of the adrenal gland, tumors of the ovary, interstitial cell tumors of the testis, teratomas of the testis, tumors of the salivary gland, and even adenomas of the Harderian gland. No doubt other tumors could be shown to be influenced by genetic factors if they could be caused to occur with such frequency as to permit a genetic study. The fact that such a list cannot be named for any other species does not indicate that the biochemistry and physiology of the mouse are more under the control of its genes, but rather that other species are not so well suited to genetic studies of cancer, or for some other reason have not been studied so much in this respect. Some tumors in which genetic influences are most pronounced occur in fruit flies, fish, and even in plants.

Influence of genetic factors is not only apparent in the development of the so-called spontaneous tumors but is equally important

strains. Obtaining significant results for these single genes with such transplantation techniques is proving difficult.

Merely localizing the action of these genes influencing the occurrence of pulmonary tumors in the lung falls far short of linking the gene to the character. However, it does suggest that the effect of these genes on the malignant change may be a rather direct one. Until we know more about the nature of the malignant change itself, however, we can hardly postulate how these genes are controlling it. Is the malignant change some heritable change in the cytoplasm; do these genes influence the probability of this change occurring, or is the malignant change a gene mutation? If it is a gene mutation, is it a mutation of a specific gene under the influence of these several genes by which the strains differ, or can it be a mutation of any of several genes? Are these postulated mutable genes themselves the genes by which the strains differ, and do those carried by the susceptible strain represent more mutable alleles than those carried by the resistant strain? These questions concern problems in the genetics of somatic cells. It can be hoped that some of the newer techniques of tissue culture or those learned in the study of microbial genetics may be applied to mammalian cells in attempting to answer such questions.

Transplantation techniques, such as those applied to the localization of the action of genes influencing the occurrence of pulmonary tumors, have been applied with general success to a number of other tumors. Male strain C3H mice do not develop interstitial cell tumors of the testis when treated with estrogen; whereas male strain A mice do Trentin and Gardner (1958) transplanted testes from males of these two strains into their castrated F_1 hybrid males, in order to ascertain whether this genetic difference represented a genetic difference in the response of the pituitary glands to the estrogen which in turn effected a difference in the response of the testis, or a difference in the direct response of the testis to the same hormonal stimulation. Pellets of diethylstilbestrol in cholesterol were then implanted in the F_1 hosts. Tumors occurred in A testes in F_1 hosts even more frequently than in A testes transplanted into A hosts, probably because of the longer life of the F_1 host, while a tumor occurred in only one of the C3H testes in F_1 hosts. This suggests that genic action here is limited to the testis and indicates that in attempting to link more completely the action of the genes to the resulting tumor one should focus his study on the testis.

It is interesting that in strain C3H males which are so resistant to estrogen-induced, interstitial cell tumors of the testis, mammary

parent strains differed by four or more pairs of genes influencing the occurrence of pulmonary tumors. If the action of these genes were limited to the lung itself, one could expect tumor development in the transplants to resemble that in the intact lungs of the respective parent strain; whereas, if the action of these genes became manifest through some systemic mechanism, one would expect the transplants to lose their respective susceptibility and resistance and respond as directed by the genotype of the F_1 host. Following the injection of the F_1 hosts with dibenz(*a,h*)anthracene to increase the tumor response and shorten the time of the experiment, many tumors were found in the strain A transplants and very few appeared in the strain C57L transplants. This result indicated that the action of these genes was localized in the end organ, the lung.

This work has been extended to similar transplantation of fetal lung (Heston and Steffee, 1957). The minute transplanted lobe of the lung of the fetus grew to a size approximating that of a corresponding lobe of an adult mouse, thus providing more tissue to respond, and hence more tumors occurred; but the transplanted lobes retained their respective susceptibility and resistance. This was observed both when the tumors were induced with dibenz(*a,h*)anthracene and when they arose spontaneously. Thus, the action of the genes in the pulmonary tissue controlling the occurrence of tumors could not be altered even in the fetal tissue.

Shapiro and Kirschbaum (1951) carried out a similar study in which segments of lung from strain DBA mice that are resistant to pulmonary tumors, and from mice of the Bagg albino strain that is susceptible, were transplanted into the ears of the F_1 hybrids of these two strains. They found that the Bagg albino transplants remained susceptible and the DBA transplants remained resistant, indicating that the action of the genes influencing the occurrence of pulmonary tumors by which these strains differed was likewise confined to the lung.

It would not be expected, however, that the action of all genes influencing the occurrence of pulmonary tumors would need to be restricted to the lung. Genes, such as lethal yellow and obese, that influence the occurrence of pulmonary tumors and also have a pronounced effect on the normal growth of the animal, might be expected to have their action influencing pulmonary tumors manifested through some general mechanism of the host. Differences due to these single genes, however, are small when compared with the large differences between the highly susceptible and the highly resistant

strains. Obtaining significant results for these single genes with such transplantation techniques is proving difficult.

Merely localizing the action of these genes influencing the occurrence of pulmonary tumors in the lung falls far short of linking the gene to the character. However, it does suggest that the effect of these genes on the malignant change may be a rather direct one. Until we know more about the nature of the malignant change itself, however, we can hardly postulate how these genes are controlling it. Is the malignant change some heritable change in the cytoplasm; do these genes influence the probability of this change occurring, or is the malignant change a gene mutation? If it is a gene mutation, is it a mutation of a specific gene under the influence of these several genes by which the strains differ, or can it be a mutation of any of several genes? Are these postulated mutable genes themselves the genes by which the strains differ, and do those carried by the susceptible strain represent more mutable alleles than those carried by the resistant strain? These questions concern problems in the genetics of somatic cells. It can be hoped that some of the newer techniques of tissue culture or those learned in the study of microbial genetics may be applied to mammalian cells in attempting to answer such questions.

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gland tumors are readily induced with estrogen; whereas the strain A males that are highly susceptible to the estrogen-induced testicular tumors are refractory to estrogen-induced mammary gland tumors (Bonser, 1944). However, this is understandable since the gene action controlling the testicular tumors controls the response of the testis to the hormonal stimulation rather than controlling the hormonal output.

There is evidence that some of the gene action influencing the occurrence of mammary gland tumors in mice is localized within the mammary gland. Prehn (1953) transplanted mammary glands from both resistant strain C57BL and susceptible strain BALB/c into the F_1 hybrid females from crosses of these two strains. Following the introduction of the mammary tumor agent, tumors and hyperplastic nodules were noted in the BALB/c transplanted glands but not in the C57BL transplanted glands. This indicated genic action within the mammary gland controlling its response but did not indicate whether it was response to the agent, to the hormonal stimulation, or to both. This area of investigation will be discussed further later.

In the classic experiments of Woolley and co-workers (Woolley, 1949) genetic differences in the occurrence of tumors in the adrenal glands associated with hormonal imbalance were demonstrated. The adrenal glands of early gonadectomized mice of strains C57BL and C57BR underwent only slight change; those of gonadectomized strain DBA mice developed nodular hyperplasia of the cortex, and those of gonadectomized strain CE mice developed carcinoma of the cortex. Was this genic action within the adrenal gland controlling its response; was the action elsewhere indirectly controlling the response of the adrenal gland; or was it controlling the abnormal hormonal stimulation to the adrenal gland? Using strain A that shows very minimal post-castrational adrenal hyperplasia, strain C3H (Z) that shows considerable hyperplasia, and strain CE that develops carcinomas, Huseby and Bittner (1951) transplanted adrenal glands from the parental strains into the various F_1 hybrid combinations of the three strains. The F_1 mice were then gonadectomized. The results indicated that the response, whether carcinoma or merely hyperplasia, was governed by the genotype of the adrenal gland. Thus, again the genic action was demonstrated to be within the end organ.

Experiments on lymphocytic leukemia in mice have yielded certain entrancing variations to the situations described above. That genetic factors are involved was clearly shown by the work of MacDowell

and co-workers (MacDowell, Potter, and Taylor, 1945) and the work of Cole and Furth (1941). Studies on thymectomy and transplantation of the thymus by Law and co-workers (Law, 1957; Law and Potter, 1956) and by Kaplan and co-workers (Kaplan, Hirsch, and Brown, 1956) implicated the thymus as the site of origin of both spontaneous and x-ray-induced lymphocytic leukemia and raised the question as to whether the genes controlling susceptibility were acting within the thymus or through some mechanism indirectly affecting the thymus. In transplantation experiments involving the F_1 hybrid Kaplan *et al.* observed that more neoplasms arose in genetically susceptible C57BL thymic transplants in irradiated (C57BL × C3H)F_1 hosts than in genetically resistant C3H thymic transplants in the same hosts. In comparison, Law observed that neoplasms arose in about half of the genetically susceptible AKR thymic transplants in (C3H × AKR)F_1 hosts, whereas none occurred in the genetically resistant C3H thymic transplants in these F_1 hosts This indicated that genic action influencing the occurrence of lymphocytic leukemia was localized in the thymus

Law and co-workers carried the study further by using genetically susceptible strain C57BL and genetically resistant strain A that have different *H-2* alleles, affording an opportunity for ascertaining whether the neoplasms actually arose from the lymphocytes introduced with the transplanted thymus or from lymphocytes of the host that invaded the transplanted thymus. They found that neoplasms arising early (five to five and one-half months) in C57BL thymic transplants in (C57BL × A)F_1 hosts were transplantable to both the parent C57BL strain and the F_1, indicating strain C57BL origin. However, those neoplasms arising in the transplants later (seven to ten months) were transplantable only to the F_1, indicating that they arose from F_1 lymphocytes Kaplan has made similar observations.

These observations, together with the observations of Law and Potter (1956) that, following transplantation, the original lymphocytes of the thymic transplant are gradually replaced by lymphocytes from the host, indicate that the genic action here could be further localized in the stroma of the thymus. It appears that gene action within the stroma, through some path which still may be rather indirect, causes the neoplastic change to occur either in the lymphocytes that accompany the thymic transplant or in host lymphocytes that later infiltrate the transplant

It should be emphasized, however, that this may not be the only genic action influencing the occurrence of leukemia. Earlier, Law

(1952) had shown that the gene, flexed-tail, was associated with an increase in induced leukemia in mice. Inasmuch as flexed-tail causes a temporary anemia at birth, one may suspect that the genic effect on the occurrence of leukemia here is not through some action limited to the stroma of the thymus but through a more general earlier effect on the whole hematopoietic system.

In addition to genic action in neoplasia being limited to certain organs or cells, sometimes the genic action is actually that of producing the cell that is to become malignant. Through extensive studies Gordon (1951) has observed that crosses between spotted platyfish and swordtails produce hybrids in which melanomas occur. From the platyfish are introduced macromelanophores and from the swordtails are introduced growth-stimulating genes that cause the macromelanophores to undergo the malignant change. The more clear-cut genetic action in this case is that resulting in the macromelanophores. These are clearly the result of any one of five sex-linked genes that are probably alleles, are dominant to the allele or alleles that do not give rise to these cells, and vary in the resultant distribution of the macromelanophores. Without the macromelanophores the melanomas cannot occur.

A somewhat comparable situation may also exist in respect to the hyperplastic nodules in the mammary glands in mice. DeOme has described the technique by which he and his co-workers have been able to show conclusively that mammary tumors arise from these nodules (see pp. 327 to 348, this volume). From the work of DeOme's group and the work of Richardson and Hummel (1959), Jones (1956), and Huseby and Bittner (1946), it is evident that the occurrence of these nodules in the various strains is largely determined by genes whose actions become manifest through hormonal stimulation. The way is open for a thorough genetic analysis of these nodules as well as an analysis of the endocrine factors involved, and I anticipate that this will be done in DeOme's laboratory. The number of nodules will undoubtedly offer a quantitative measure of response that will be a great aid in the genetic analyses, just as the number of induced pulmonary tumors in mice has proved to be such a valuable measure of response in the genetic analysis of pulmonary tumors.

Sometimes gene action influencing the occurrence of tumors can be localized physiologically rather than anatomically. For some time we have been interested in the relationship between normal growth and malignant growth. Among seven genes shown to be associated with pulmonary tumors in mice (hairless, lethal yellow, vestigial tail,

shaker-2, waved-2, fused, and flexed-tail) there has appeared to be a positive correlation between the effect of these genes on body weight and their association with the occurrence of pulmonary tumors (Heston, Deringer, Hughes, and Cornfield, 1952; Deringer and Heston, 1955; Heston, 1956). Lethal yellow that increases body weight increased the occurrence of pulmonary tumors, whereas all the other genes were associated with a decrease in pulmonary tumors, and all decrease body weight. This suggested that the action of at least some of these genes on the occurrence of tumors might be linked in some way to their effect on body weight.

More recently, however, we have found a decided decrease in occurrence of pulmonary tumors associated with the gene obese, and this gene greatly increases body weight (unpublished data). Although this at first appeared contrary to results with the other genes, further study of the obese animals revealed that although they weighed approximately twice as much as their normal sibs because of the great amount of adipose tissue, they were smaller than their normal sibs in respect to measurements taken on total body length, length of the femur, and weight of gastrocnemius muscle. Lethal yellow that increases occurrence of tumors not only increases body weight but also increases total body length and length of the femur These observations suggest that occurrence of pulmonary tumors is not genetically related to obesity but rather to normal growth of the skeletal and muscular elements.

In this connection it is interesting to note that from preliminary observations on the relationship of body types to occurrence of breast cancer in women, Sheldon and associates (1949) reported that those most likely to develop breast cancer were women with good muscular and skeletal development as well as some tendency toward obesity, but the very obese women who obviously had endocrine disturbances and were low on the mesomorphic scale were much less likely to develop breast cancer. These results together with those on the specific genes in mice suggest that a more fruitful approach to the discovery of genetic effects on cancer in man might be directed toward the study of genes affecting normal growth as revealed in body types rather than toward a search for linkage with those genes influencing more superficial characters such as hair color or eye color.

Wolff (1956) has started some biochemical studies of the effect of the lethal yellow gene. Since Greenstein (1942) had shown some differences between normal and neoplastic mouse lung in respect to phosphatase activity, Wolff decided to analyze precancerous lung

homogenates from yellow and non-yellow mice in this respect, and thus far has found some differences that appear to be associated with this gene. Much more work will need to be done before the picture is clarified and before the biochemical differences can be related to the effect of the gene on either normal or malignant growth. The work, however, represents an example of a start in the biochemical genetics of the mouse, an area that must be developed if genic action is to be completely linked with neoplasia in this species.

Some examples of genetic relationship between normal and malignant growth can be found in the field of plant tumors. After studying 31 interspecific tumor-forming hybrid combinations in Nicotiana, Kehr (1951) concluded that these tumors were caused by genes controlling the growth-regulating mechanism. When this mechanism is thrown out of balance in plants of certain genetic constitutions, the tumors occur. He postulated that whereas the species comprise systems in which phytohormone utilization and other developmental processes result in normal growth, in certain interspecific combinations the resultant genotype disturbs the normal phytohormone metabolism, resulting in abnormal growth without normal cell differentiation.

In some types of cancer, genes influence the occurrence of tumors through their control of response of certain tissues to some external stimulus. This also might be thought of as physiological localization of gene action. One of the cancers of man exhibiting a rather simple mode of inheritance is xeroderma pigmentosum. Macklin (1936) described the inheritance as that of a simple recessive trait. Before the mode of inheritance was known, however, actinic rays had been established as a causative factor (Copeland and Martin, 1932). Upon exposure to sunlight, genetically susceptible individuals develop an intensive freckling that progresses to a carcinomatous condition. It is to be assumed that the actinic rays cause a heritable change or heritable changes in the cell resulting in malignancy, and that the action of the gene is that of greatly increasing the probability that this change or these changes will occur.

The bovine ocular squamous carcinoma that is being studied by staff members of The University of Texas M. D. Anderson Hospital and Tumor Institute may be the result of similar gene action (Anderson, see pp. 364 to 374, this volume; Russell, Wynne, and Loquvam, 1956). The gene or genes involved here may be increasing the probability that the squamous epithelial cells in the ocular region will undergo a heritable malignant change when exposed to sunlight.

Sometimes the gene action can be localized physiologically in the production of the stimulus necessary for the tumor to occur when this stimulus is intrinsic. Very few virgin strain A female mice develop mammary gland tumors; whereas mammary gland tumors occur in practically all virgin as well as breeding strain C3H females. If strain A females are bred, however, an incidence approaching that in C3H females is produced. Through crosses between these strains this difference was shown to be basically due to genes (Bittner, Visscher, Ball, and Smith, 1944; Heston and Andervont, 1944). The question remained, however, as to whether the genic action was localized in the physiology of hormone production, causing the C3H to produce hormonal stimulation necessary for the females to have tumors without the added hormonal stimulation resulting from having litters; or was localized in the mammary gland, making the C3H glands more responsive. Huseby and Bittner (1948) have answered this question through transplantation of ovaries. When they transplanted A, C3H, and (C3H × A)F_1 ovaries into spayed (C3H × A)F_1 females they observed that the F_1 females bearing C3H and F_1 ovaries had a higher mammary tumor incidence with a lower tumor age than did the F_1 females bearing A ovaries. These results indicated that the action of some of the genes by which the strains differed was localized in the physiology of hormone production by the ovary. There was a suggestion, however, that other genes might be controlling the response of the mammary tissue to the hormonal stimulation from the ovary, because spayed F_1 females bearing transplanted A ovaries had a somewhat higher incidence of mammary tumors than did spayed A females bearing transplanted A ovaries The excellent technique of transplanting hyperplastic nodules and normal mammary gland, described by DeOme, will be well suited to yielding further information on this second point.

Finally, gene action in the occurrence of tumors can be localized in host-parasite relationships where microorganisms are involved in the induction of the tumor. In respect to tumor viruses, one can expect genetic differences in the hosts resulting in variation in response to the virus as well as genetic differences in the viruses Thus far, however, genetic variations in the hosts have probably received greater attention. For example, in 1957, Bryan reported strain differences in the response of chickens to the Rous sarcoma virus. Through inbreeding and selection, Waters (1945) developed lines of chickens resistant and susceptible to the lymphomatosis virus.

The wound tumor virus in sweet clover presents an excellent

homogenates from yellow and non-yellow mice in this respect, and thus far has found some differences that appear to be associated with this gene. Much more work will need to be done before the picture is clarified and before the biochemical differences can be related to the effect of the gene on either normal or malignant growth. The work, however, represents an example of a start in the biochemical genetics of the mouse, an area that must be developed if genic action is to be completely linked with neoplasia in this species.

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The wound tumor virus in sweet clover presents an excellent

example in plants of genetic control of susceptibility to the tumor-inducing virus. This has been studied extensively by Black and coworkers (Black, 1951; Littau and Black, 1952), who have postulated that many genes are involved. Genes not only determine susceptibility and resistance, but also determine number and size of tumors induced, and whether the tumors are located on the stems, on the roots, or on both. The paths of gene action appear to be within the cell and not hormonal, for a susceptible root was not rendered resistant by growing it on a top from a clone having a resistant root and vice versa. Of special side interest is the fact that genetically highly susceptible plants will develop some tumors without the introduction of the virus, which parallels the situation in respect to mammary gland tumors in mice.

In 1945 we published evidence of a definite genic control over the propagation and transmission of the mammary tumor agent or virus in mice (Heston, Deringer, and Andervont, 1945). Genetically susceptible strain C3H females with the agent were outcrossed to genetically resistant C57BL males without the agent. The F_1 females were then backcrossed to C3H males to produce a group of C3H backcross females and to C57BL males to produce a group of C57BL backcross females. Although both groups of backcross females had received similar agent because of the homogeneity of the F_1 mothers, the C3H backcross females transmitted the agent much more than did the C57BL backcross females. Uniform test females were used, and those that nursed upon the C3H backcross females had a higher incidence of mammary tumors than did those that nursed upon the C57BL backcross females. This difference between the two backcross groups in ability to propagate and transmit the agent was due to genetic differences resulting from one group having had C3H fathers and the other group having had C57BL fathers.

In a later, more extensive study (Heston, Deringer, and Dunn, 1956), it was found that by outcrossing strain C3H females to strain C57BL males and following this with successive backcrosses of the hybrid females to C57BL males, the agent was completely eliminated by the third backcross generation because of the concentration of the resistant C57BL chromatin. This early elimination of the agent suggested that, contrary to our original assumption of many genes controlling the agent, they were probably few in number and possibly there was only a single pair. We hope to have more information in the near future on the number of genes involved.

We are still not in position to explain just how this gene or these

genes act in controlling this agent or virus, but the indication of relatively few genes with a rather direct control over the virus suggests that the problem may not be as complex as originally assumed. A recent observation by Tjio and Östergren (1958) of preparations of chromosomes of mammary gland tumors provides ground for some speculation. In a series of 19 spontaneous mammary gland tumors from strains C3H and DBA with the agent, they observed in each of 16 diploid tumors one heteropyknotic chromosome. In one tetraploid tumor and in one tetraploid and diploid tumor, there were two such heteropyknotic chromosomes in the tetraploid cells. In one tumor with only 39 chromosomes, the heteropyknotic chromosome was not observed This is one of the most uniform and possibly one of the most important chromosome changes that has been observed in tumors. If it could be shown that this heteropyknotic chromosome was always the same chromosome and that it was the result of the mammary tumor agent, some phenomenon similar to the integrated stage in lysogeny, with the gene or genes that control the agent causing this chromosome to be the susceptible one, would be suggested. Many questions must be answered, however, before such a conclusion can be reached

The paths linking the primary actions of genes to the various types of cancer are complex and interwoven chains of events. On the levels of the organism as a whole, of specific organs, and of tissues, considerable progress has been made in identifying links toward the ends of chains leading to several types of cancer. With present materials and techniques, further progress in this area can be expected. Identification of the links nearer the primary gene action must be made on the cellular and subcellular levels. With the advancement of such areas as gene chemistry and biochemical genetics of the mammal, and with the application of some of the advances in tissue culture and some of the techniques of microbial genetics to the mammalian cell, progress in the identification of the early links can be expected, with the ultimate goal of finally linking the gene to the cancer.

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Cytogenetics of Experimental Tumors

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It is generally realized that malignancy is a composite phenomenon, including several different biological properties, or, in the terminology of Foulds (1954, 1958), unit characters. These characters usually are observed at the population level and can be exemplified as: growth rate, dependence on hormonal controls, invasiveness, metastasizability, synthesis of differentiation products demonstrable by morphological, biochemical, or functional examination, the ability to grow in the ascites form, sensitivity to antimetabolites and other growth inhibitors, etc. Consideration of the natural history of various forms of cancer indicates that tumors often develop by a series of independent changes of several unit characters. This process, termed "tumor progression," is reminiscent of the development of certain microorganismal populations by a series of adaptive changes In a broad sense, progression moves in one general direction and molds tumors into new forms that are increasingly independent of superimposed control mechanisms of the organism The detailed course of progression is extremely variable, however. In the words of Foulds (1958): "The 'essential malignancy,' 'biological potential,' or 'growth pattern' of a tumor depends upon a particular association of unit characters. The possible combinations of unit characters are extremely numerous so that each tumor is a particular entity differing from all others." In the view of Huxley (1956), "all autonomous neoplasms can be regarded as the equivalents of new biological species."

Workers interested in developing the field of tumor cytogenetics—and this field is presently in its embryonic stage at best—are naturally attracted by these phenomena, and the opinion has often been voiced that progression may have a classical mutation-selection basis. While this is possible and, in some instances at least, highly probable, the situation as a whole may be more complicated in reality. Somatic cells of higher organisms are subject to developmental processes. Unfortunately, differentiation and related phenomena are still among the most obscure in biology, but it is probable that they are not mutational in nature; i. e., are not based on random changes of structurally *coded information transmitted from cell to cell in connection with* division. Since the normal processes are poorly defined and only vaguely known in terms of cellular mechanisms, it is difficult to guess to which group progression phenomena in tumors might belong.

These difficulties should not lead to nihilism, but they justify an approach free of too many theoretical preconceptions and focused on the thorough experimental analysis of relatively simple situations. Generalizations from such analyses will seldom be justified except in the form of working hypotheses. What are the possibilities, then, of designing experimental systems for such an analysis, how much can be learned about the nature of the changes observed, and what is their significance for the understanding of tumor formation and the subsequent evolution of neoplastic cell populations?

Since somatic cells are presently unavailable for sexual recombination or other forms of genetic transfer, their straightforward genetic analysis is not possible. Indirect methods of various kinds may nevertheless yield certain information; a recent symposium entitled "Genetic Approaches to Somatic Cell Variation" (Hollaender, 1958) gives a good survey of such experiments.

In the particular case of tumor cells, the two main approaches hitherto employed can be registered as *the study of phenotypic* marker characteristics and the detailed examination of chromosome morphology. This paper will deal mainly with the former approach. To restrict the subject matter further, only *in vivo* systems will be discussed.

Marker Characteristics

Several considerations enter into the selection of suitable marker characteristics. It is desirable to have a fair knowledge of their genetic determination mechanism This should be sufficiently variable in nature to permit the identification of a series of alternative

forms. A selective device of some kind must be available that permits the identification and concentration of rare alternative forms ("variants") from large cell populations.

Among the phenotypic characteristics encountered in neoplastic cell populations *in vivo,* there are some that are common to most or all normal and neoplastic cells of the organism, while others are more or less distinctive of the neoplastic transformation itself. The former category comprises the isoantigens of the histocompatibility system. These have been explored in considerable detail by several schools of transplantation geneticists, immunologists, and general biologists, among whom Little (1941), Strong (1922), Snell (1953), Gorer (1956), Hoecker (1956), Amos (1956), Medawar (1957) and their co-workers can be mentioned. It is neither necessary nor feasible to discuss these developments here; several excellent review articles of recent date are available on the subject (Brent, 1958; Gorer, 1956; Hauschka, 1952, 1957; Medawar, 1957; Snell, 1953, 1957). Since these characteristics are common to all cells of the organism, they can be subjected to straightforward genetic analysis by crosses; and their genetic determination mechanism is fairly well known, at least in the mouse Other species are being studied to an increasing extent (Favour, 1958; Hildemann, 1957; Kallman and Gordon, 1958).

The second category of properties corresponds to the "unit characters" mentioned in the previous paragraph; their determination mechanism is unknown. They are nevertheless of great interest because of their direct relationship to malignancy. Since they change independently during progression and their assortment is more or less random in the final tumor, it must be postulated that they are not necessarily interrelated and are probably determined by different cellular mechanisms

The selectivity of the systems involved has not been studied to a very considerable extent; some reconstruction experiments with artificial mixtures of different cell types are available with the histocompatibility system (G Klein and E. Klein, 1956), some cases of drug resistance in mouse leukemia (G. Klein and E. Klein, 1957; G. Klein, 1959, Potter, 1959), and the convertibility of a certain solid mouse sarcoma into the ascites form (G Klein and E. Klein, 1955). Among these, only the first permits the design of absolutely selective systems where the selective force utilized (the homograft reaction) is able to inhibit the growth *of one cellular* phenotype completely while leaving an alternative form undamaged. In the other two systems, growth inhibition of one type is not complete, and the selective ad-

Workers interested in developing the field of tumor cytogenetics—and this field is presently in its embryonic stage at best—are naturally attracted by these phenomena, and the opinion has often been voiced that progression may have a classical mutation-selection basis. While this is possible and, in some instances at least, highly probable, the situation as a whole may be more complicated in reality. Somatic cells of higher organisms are subject to developmental processes. Unfortunately, differentiation and related phenomena are still among the most obscure in biology, but it is probable that they are not mutational in nature; i. e., are not based on random changes of structurally coded information transmitted from cell to cell in connection with division. Since the normal processes are poorly defined and only vaguely known in terms of cellular mechanisms, it is difficult to guess to which group progression phenomena in tumors might belong.

These difficulties should not lead to nihilism, but they justify an approach free of too many theoretical preconceptions and focused on the thorough experimental analysis of relatively simple situations. Generalizations from such analyses will seldom be justified except in the form of working hypotheses. What are the possibilities, then, of designing experimental systems for such an analysis, how much can be learned about the nature of the changes observed, and what is their significance for the understanding of tumor formation and the subsequent evolution of neoplastic cell populations?

Since somatic cells are presently unavailable for sexual recombination or other forms of genetic transfer, their straightforward genetic analysis is not possible. Indirect methods of various kinds may nevertheless yield certain information; a recent symposium entitled "Genetic Approaches to Somatic Cell Variation" (Hollaender, 1958) gives a good survey of such experiments.

In the particular case of tumor cells, the two main approaches hitherto employed can be registered as the study of phenotypic marker characteristics and the detailed examination of chromosome morphology. This paper will deal mainly with the former approach. To restrict the subject matter further, only *in vivo* systems will be discussed.

Marker Characteristics

Several considerations enter into the selection of suitable marker characteristics. It is desirable to have a fair knowledge of their genetic determination mechanism. This should be sufficiently variable in nature to permit the identification of a series of alternative

TABLE 1 Summary of Transplantation Tests with *Tumors* Originating in Homozygotes

Tumors, No. and type	*Genotype of* origin	No. mice killed by progressively growing tumors/No. mice inoculated				
		A/Sn	A.SW	A.BY	A.CA	Various F_1 comb. between *two* foreign IR-lines
5 MC sarcomas	A/Sn	41/44	0/25	0/22	0/18	0/26
3 MC sarcomas	A.SW	0/21	17/19	1/15(1 fp)	0/15	0/15
5 MC sarcomas	A.CA	1/62(1 nsp)	3/54(2 fp)	0/54	58/61	2/36(1 fp)
1 spontaneous mammary carcinoma	A/Sn	10/10		0/6	0/5	
1 spontaneous mammary carcinoma	A.SW	0/12	13/13	0/8	0/8	0/15
1 spontaneous mammary carcinoma	A.CA	0/3	0/3		2/3	

Abbreviations used in Table 1:

fp, *false positives*
MC, methylcholanthrene-induced
nsp, nonspecific forms, growing in a wide variety of unrelated genotypes

vantage of deviating variants is relative rather than absolute. This makes them less selective; in the particular cases investigated by reconstruction experiments, the minimum frequency of demonstrable variants has been of the order of 10^{-6} in the case of amethopterin resistance (G. Klein and E. Klein, 1957) and 4×10^{-5} with the ascites system (G. Klein and E. Klein, 1955).

Isoantigenic Analysis

Since antigens are believed to reflect the specificity of genes more directly than other cellular characteristics (Wright, 1945) and, further, because of the known genetic determination mechanism of the isoantigens of the histocompatibility system of the mouse and the availability of highly selective experimental model systems, more detailed consideration will be given to some of our experiments dealing with the isoantigenic analysis of certain tumor cell populations.

We have been particularly interested in tumors arising in heterozygous F_1 hybrid hosts, produced by the crossing of two of the isogenic resistant (IR) mouse lines of Snell (1948, 1958b). The use of such material for this purpose has been discussed by Lederberg (1956), and a number of studies have been published previously by Mitchison (1956, 1958) and by our group (G. Klein and E. Klein, 1956; E. Klein, G. Klein, and Révész, 1957; G. Klein and E. Klein, 1958; Bayreuther and E. Klein, 1958). The IR-lines have been bred in order to establish a coisogenic background while maintaining an allelic difference at one of the histocompatibility loci. Theoretically, they are homozygous with regard to their entire genome with the exception of the histocompatibility gene in question and a chromosome section of undefined length around it. It is difficult to say how well this theoretical expectation is fulfilled in reality; the existing IR-lines are probably good approximations only. Provided that the number of major histocompatibility genes differentiating the two lines used for the cross were not more than one, tumors induced in the F_1 hybrids would be particularly suitable for detection of isoantigenic variation at the cellular level. They might be expected to give rise to variant cells, selectively compatible with one or the other of the parental strains, either by mutation or as a result of phenotypic changes involving the inactivation or loss of the isoantigenic components specifically derived from the opposite parental strain.

We have tested a considerable number of induced or spontaneous tumors of such F_1 hybrids, in order to study the possibility of extracting new and specific isoantigenic variants by transplantation to ap-

6 MC sarcomas	A CA♂ x A BY♀ F_1	71/77 (92%)			1/108 (0 9%)	10/102(1 fp) (10%) (1 var 2)
4 MC sarcomas	A SW♂ x A CA♀ F_1	46/47 (98%)	0/22	7/111(2 nsp) (6%) (1 var 2)	0/25	11/106(1 nsp) (10%) (1 var 1) (2 var 2)
6 MC sarcomas	A CA♂ x A SW♀ F_1	93/97 (96%)		13/128(1 var 1) (10%) (3 var 2)		20/119(2 var 2) (17%)
2 MC sarcomas	A♂♀ x A BY♀♂ F_1	49/54 (91%)	8/90(1 var 1) (9%)	1/12(1 fp)	13/90(1 var 2) (14%)	
4 MC sarcomas	A SW♂♀ x A BY♀♂ F_1	34/34 (100%)	0/38	20/57(2 nsp) (35%)	4/47(1 nsp) (9%)	0/23

Abbreviations used in Table 2

fp, false positives
MC, methylcholanthrene-induced
nsp, nonspecific forms, growing in a wide variety of unrelated genotypes
var 1, variants, specifically compatible with nonimmunized mice of the same genotype
var 2, variants, specifically compatible with both untreated and preimmunized mice of the same genotype
var 1-2, same as var 1, but changing to var 2 after a variable number of passages in nonimmunized hosts

TABLE 2 Summary of Transplantation Tests with Methylcholanthrene-Sarcomas Originating in Heterozygotes

Tumors, No and type	Genotype of origin	No. mice killed by progressively growing tumors/No. mice inoculated				
		Takes in F_1 type of origin	A/Sn	A SW	A.BY	A.CA
13 MC sarcomas	A♂ x A SW♀ F_1	380/383 (99%)	29/410(1 fp) (7%) (2 var 1) (1 var 2) (4 nsp)	192/898(1 fp) (21%) (4 var 1) (1 var 2) (4 nsp)	0/48	0/29
9 MC sarcomas	A♀ x A SW♂ F_1	150/162 (93%)	27/221(3 fp) (12%) (1 var 1) (1 nsp)	36/248(1 fp) (15%) (1 var 1) (1 var 1–2) (6 var 2) (1 nsp)		
6 MC sarcomas	A♂ x A CA♀ F_1	91/123 (74%)	11/136(1 var 1) (8%) (3 var 1–2)	2/79 (1 nsp)	0/49	18/93(1 fp) (19%) (6 var 2)
7 MC sarcomas	A.CA♂ x A♀ F_1	100/105 (95%)	32/135(2 fp) (24%) (4 var 2)			47/149(5 var 2) (31%)
8 MC sarcomas	A BY♂ x A CA♀ F_1	144/146 (99%)	0/7		15/227(2 fp) (7%) (1 var 2) (1 nsp)	88/264(2 fp) (33%) (1 var 1) (3 var 2) (1 nsp)

TABLE 3 Summary of Transplantation Tests with Lymphomas and Carcinomas Originating in Heterozygotes

Tumors, No. and type	Genotype of origin	No. mice killed by progressively growing tumors			No. mice inoculated	
		Takes in F_1 type of origin	A/Sn	A.SW	A.BY	A.CA
2 mammary carcinomas	A♂ x A.SW♀ F_1	10/12	1/5	0/8		
3 mammary carcinomas	A♂ x A.CA♀ F_1	23/29	1/20(1 var 2)		0/3	0/24
1 mammary carcinoma	A.BY♂ x A.CA♀ F_1	30/34			0/14	0/10
2 lymphomas	A♂ x A.SW♀ F_1	74/75	6/93(1 var 1–2)	16/93(2 var 1–2) (1 var 2)		

Abbreviations used in Table 3

var 1, variants, specifically compatible with nonimmunized mice of the same genotype
var 2, variants, specifically compatible with both untreated and preimmunized mice of the same genotype
var 1–2, same as var 1, but changing to var 2 after a variable number of passages in nonimmunized hosts

propriate host genotypes. The variant lines obtained were characterized by further transplantation tests, by serological determination of known isoantigens of the H-2 system, and by morphological examination of their chromosomes. All tumors have been tested at the primary stage and during their first eight or ten transfer generations. The data obtained until about a year ago have been published elsewhere (G. Klein and E. Klein, 1958; Bayreuther and E. Klein, 1958). Since then, an extensive body of new data has been accumulated. It would be impossible to enter here into all details, and attention will be focused on a few essential points only.

Specific Variants, False Positives, and Nonspecific Forms

Occasional tumor takes obtained in hosts other than the genotype of origin were tested further by transplantation to members of the same foreign genotype and to other foreign genotypes. Different types of behavior could be observed. For simplicity, those that did not grow on repeated testing in the same foreign genotype will be referred to as "false positives"; those that grew in most or all mice of the foreign genotype in question but in no other foreign genotypes will be called "specific variants" or, simply, "variants"; and those that grew in addition in a wide variety of other foreign genotypes will be denoted as "nonspecific" forms. The mouse strains used were four isogenic resistant (IR) lines of Snell (1958b): A/Sn ($H\text{-}2^aH\text{-}2^a$), A.SW ($H\text{-}2^sH\text{-}2^s$), A.BY ($H\text{-}2^bH\text{-}2^b$), and A.CA ($H\text{-}2^fH\text{-}2^f$).

Specific variants can only be obtained from heterozygous tumors and only in one of the parental strains. Of 16 tumors of homozygous origin that were tested (Table 1), very few takes were obtained outside the genotype of origin. Whenever tested, these turned out to be false positives or nonspecific forms. However, several heterozygous F_1 hybrid tumors gave rise to a certain number of takes in one or the other parental strain (Tables 2 and 3). While some of these proved to be false positives and others were nonspecific, a fairly high proportion turned out to be specific variants, capable of breeding true in the same parental genotype upon repeated testing, and still refusing to grow in the opposite parental type or in other, unrelated genotypes. No specific variants were obtained in foreign genotypes; all such takes, when tested, turned out to be false positives or nonspecific forms, similar to the situation with homozygous tumors. Neither was it possible to obtain variants specifically and exclusively compatible with another F_1 hybrid type which was derived by crossing one of the parental types with another, foreign genotype.

TABLE 3 Summary of Transplantation Tests with Lymphomas and Carcinomas Originating in Heterozygotes

Tumors, No. and type	Genotype of origin	No. mice killed by progressively growing tumors: Takes in F_1 type of origin	A/Sn	A.SW	No mice inoculated: A.BY	A.CA
2 mammary carcinomas	A♂ x A.SW♀ F_1	10/12	1/5	0/8		
3 mammary carcinomas	A♂ x A.CA♀ F_1	23/29	1/20(1 var 2)		0/3	0/24
1 mammary carcinoma	A.BY♂ x A.CA♀ F_1	30/34			0/14	0/10
2 lymphomas	A♂ x A.SW♀ F_1	74/75	6/93(1 var 1–2)	16/93(2 var 1–2) (1 var 2)		

Abbreviations used in Table 3:

var 1, variants, specifically compatible with nonimmunized mice of the same genotype
var 2, variants, specifically compatible with both untreated and preimmunized mice of the same genotype
var 1–2, same as var 1, but changing to var 2 after a variable number of passages in nonimmunized hosts

Variant formation can proceed in several distinct steps. In agreement with the findings of Mitchison (Hollaender, 1958, p. 160), we have obtained a number of variants from different F_1 tumors that grew in all untreated mice of one parental strain ("var 1" in Table 2), but regressed if these hosts were preimmunized against the isoantigens of the opposite parent. There were other variants, obtained with other tumor-host combinations, that grew in preimmunized mice immediately on their first testing ("var 2" in Table 2). In most cases, "var 1" types were carried for several serial passages in untreated mice and were also tested in preimmunized mice on each transfer. It often happened that they changed and became able to grow in preimmunized mice also after one or several such transfers (cf. "var 1–2" in Table 2). This suggests that the number of histocompatibility barriers separating the two IR-lines entering these crosses may be more than one, since weak barriers are often not expressed unless the hosts have been preimmunized (Amos, Gorer, and Mikulska, 1955; Snell, 1958a). It appears, furthermore, that the establishment of variants fully compatible with preimmunized parental mice may in certain cases occur in at least two, and possibly more, distinct steps.

To investigate this possibility, we have been looking for a better defined system that could be analyzed in terms of known isoantigenic components. Such systems can be designed within the limits provided by the known isoantigens of the compound H-2 locus alone. For example, the isoantigenic composition of A × A.SW F_1 hybrids, determined by H-2, is $\frac{CDEFKH}{CEFGS}$; the corresponding situation for A.SW × DBA F_1 would be $\frac{CEFGS}{CDE^dFH}$; and for A.SW × C3H F_1 it would be $\frac{CEFGS}{CEHK}$ (Hoecker, 1956). Upon inoculation of tumors induced in A×A.SW F_1 mice, A.SW×DBA hybrids would therefore respond with anti-K and A.SW × C3H mice with anti-D antibodies. We have been attempting to establish variants of A × A.SW F_1 tumors specific for A SW × DBA F_1 and presumably lacking K and others specific for A.SW × C3H F_1, presumably lacking D, in order to see whether each of these changes could be followed by the second after appropriate selective passage. These experiments have been performed with only one tumor until present (the MSWB sarcoma). It turned out to be possible to establish variants compatible with A.SW

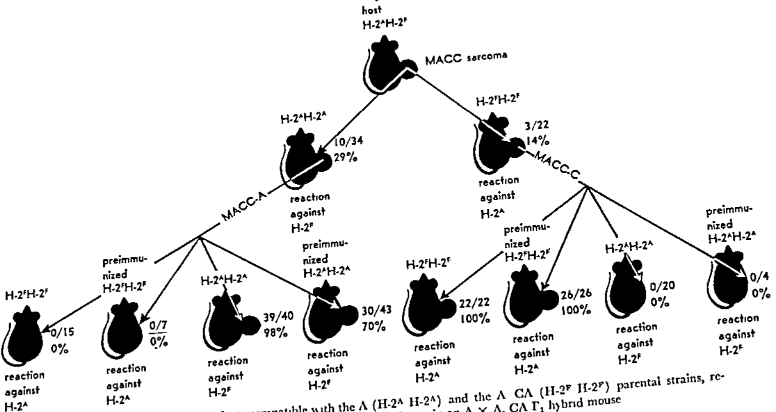


Figure 1 Establishment of variants compatible with the A ($H\text{-}2^A$ $H\text{-}2^A$) and the A. CA ($H\text{-}2^F$ $H\text{-}2^F$) parental strains, respectively, from the MACC sarcoma, induced by methylcholanthrene in an A × A. CA F_1 hybrid mouse

×DBA mice even if the hosts were preimmunized against the original line of the tumor, but these variants refused to grow in more than a limited percentage of A.SW × C3H mice. By subsequent selective passage through A.SW × C3H, these variants could be adapted secondarily to A.SW × C3H also. Variant formation could thus be broken up into two distinct steps. Oddly enough, the reverse experiment did not work. Whenever variants were selected for compatibility with A.SW × C3H directly from the original tumor, they were always automatically compatible with A.SW × DBA also. If these results are tentatively interpreted in serological terms, they indicate that while component K can be lost from this tumor either alone, or together with D, loss of D is always accompanied by loss of K. It remains to be seen whether this behavior is specific for the MSWB tumor or has more general validity.

Variants compatible with A.SW×C3H often showed the behavior discussed in the beginning of this section. They grew at first only in untreated mice and acquired the ability of growing in preimmunized mice only at a later stage, after one or several passages in untreated mice. This indicates that the tumor cells have to transgress several minor isoantigenic barriers, not expressed in untreated mice, before they can achieve full compatibility with their new hosts.

Different types of variants can be obtained from the same F_1 tumor. This has been illustrated already. It can be emphasized particularly that it was possible to establish both main forms of variants from many tumors; i.e., variants that were selectively compatible with either the maternal or the paternal strain and refused to grow in the opposite parent. This is illustrated in Figure 1 for the MACC sarcoma. In addition, the previous paragraph illustrates that it may be possible to break down the establishment of a given parental variant into intermediate steps. It has to be pointed out, however, that all variants hitherto obtained behave as if they lack some H-2-determined isoantigenic component(s) in relation to the original line of the tumor. To date, no variants could be extracted that would have been specifically and selectively compatible with a host that contains H-2 isoantigens not possessed by the original genotype of the tumor. For instance, in spite of many trials with a series of sarcomas of A × A.SW F_1 hybrid origin, we were unable to obtain variants exclusively compatible with A × A.BY, A × A.CA, A.SW × A.BY or A.SW × A.CA F_1 hybrids. Of course, all variants compatible with any of the parental strains grew also in all F_1 hybrids where the same parental strain entered the cross, but they were not specifically com-

patible with any one of these hybrids to the exclusion of the parental strain and the other hybrids. Variants of the latter type could only arise if a specific gain of an isoantigen were to occur simultaneously with the loss of another, alternative isoantigen (Table 4). If variant formation is of simple mutational origin, it is difficult to see why

TABLE 4 Variants Obtained from Tumors of A × A.SW F_1 Hybrid Origin as Compared with Minimum Changes Required in the H-2 System

Selective host	Minimum isoantigenic change required in specific variants	Specific variant formation
A.SW	Loss of D+ K+ H	+
A.SW × DBA F_1	Loss of K	+
A.SW × C3H F_1	Loss of D	–
	(Loss of D+ K)	(+)
A.SW × A.BY F_1	Loss of D+ K+ H+ *Gain of B*	–
A.SW × A.CA F_1	Loss of D+ K+ *Gain of I*	–
A	Loss of G+ S	+
A × A.BY F_1	Loss of G+ S+ *Gain of B*	–
A × A.CA F_1	Loss of S+ *Gain of I*	–

such a change should not occur. The same result also would be expected if variant formation were due to an exchange of genetic fragments between tumor and host cells, in analogy with the phenomenon of transduction (cf. Hollaender, 1958, p. 165).

Permanence of Variant Characteristics after Return to the Original Host Genotype

A number of representative variant lines have been returned from their selective hosts to the original F_1 hybrid type and were carried there continuously for 10 to 15 serial passages. In several instances, this was done after only a single cycle of growth in the former host. Subsequently they were tested again in the selective hosts. In all cases studied, they maintained their variant characteristics in spite of their prolonged sojourn in the original host environment. In fact, variants corresponding to the definition of "var 1" (cf. Table 1) were unchanged with regard to their inability to grow in preimmunized hosts, while other variants that have descended directly from the same

MSWB
sarcoma
($A \times A.SW\ F_1$)

SC : $A.SW \times C3H\ F_1$, untreated

ISC : $A.SW \times C3H\ F_1$, preimmunized against A

AS : $A \times A.SW\ F_1$

Figure 2 Permanence of variant characteristics after return to the original host genotype The MSWB sarcoma (of $A \times A$ SW F_1 origin) has been used Double circles indicate passage through the selective A. SW $\times$ C3H F_1 host Figures indicate the number of mice killed by progressively growing tumors/number of mice inoculated

"var 1" but have become "var 2" after additional selective passages, maintained their full ability to grow in preimmunized hosts. One such experiment is illustrated in Figure 2. It must be concluded that variant formation is a process that cannot be readily reversed by returning the tumor into its original host genotype.

Isoantigenic Characterization of the Variants

The question arose whether the changed transplantation behavior of the variants was really due to loss or inactivation of the relevant isoantigens of the H-2 system. This was tested in a number of different ways. The original tumor and its variant(s) were compared (E. Klein, G. Klein, and Révész, 1957; G. Klein and E. Klein, 1958; Bayreuther and E. Klein, 1958) for their ability to induce the formation of hemagglutinins (Gorer and Mikulska, 1954) and cytotoxic antibodies (Gorer and O'Gorman, 1956) directed specifically against the H-2-determined isoantigenic products of both parental strains; for their ability to absorb preformed hemagglutinins or cytotoxic antibodies from isoimmune sera; and for their ability to provoke a "second-set response" subsequent to the inoculation of heavily irradiated cells into the variant-compatible type, tested by challenging the pretreated mice with another F_1 hybrid tumor derived from the same genotype and capable of temporary growth in the parental type in question (E. Klein, 1959). In addition, our co-worker K. E. Hellstrom (1959) has studied the sensitivity of F_1 hybrid lymphomas and their variants toward various isoimmune sera by the direct technique of Gorer and O'Gorman (1956).

All techniques gave essentially similar results. Variants which were able to grow in a large number of hyperimmunized mice of their new parental genotype did not seem to contain detectable amounts of isoantigens derived specifically from the opposite parental strain. Less clear-cut results were obtained with variants that grew in nonimmunized but not in preimmunized mice. In several cases, opposite parental antigens were still detectable by the sensitive method of provoking a second-set response with preirradiated cells and by the direct cytotoxic test on the lymphoma cells. In two cases, subsequent serial transplantation in nonimmunized mice of the parental genotype led to an increased ability of such variant cells to grow in preimmunized mice. When tested again at this stage, the specific antigens of the opposite parental strain were no longer detectable with either technique.

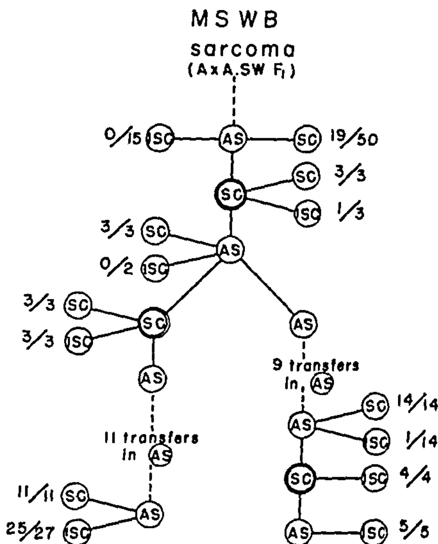


Figure 2 Permanence of variant characteristics after return to the original host genotype The MSWB sarcoma (of A × A SW F_1 origin) has been used Double circles indicate passage through the selective A SW × C3H F_1 host Figures indicate the number of mice killed by progressively growing tumors/ number of mice inoculated

strength, making the development of compatibility with one parental strain less likely than with the other. If the barriers are different in number, a greater number of changes has to occur in one direction than in the other. The finding that variants develop by at least two steps in a number of cases, enabling growth first in untreated and only later in preimmunized mice, can be interpreted to mean that several barriers have to be transgressed. If the barriers are different in strength, the periods of "background growth" of the original, incompatible tumor, previous to its regression, may be of different length. The importance of background growth for variant formation is indicated by the findings of Mitchison (1958), confirmed by us, showing that one passage through newborn mice may facilitate the extraction of variants growing in nonimmunized mice in certain cases. Analogously, passage through nonimmunized mice facilitates the establishment of variants compatible with preimmunized mice. Furthermore, in all three crosses where strain A entered (A × A.SW, A × A BY, and A × A CA), it was always the less frequented parental type with respect to variant formation (G. Klein and E. Klein, 1958). It may be recalled that the three isogenic resistant strains A.SW, A.BY, and A CA have all been derived from crosses of strain A with another, unrelated strain (Snell, 1948, 1958b). This was followed by repeated backcrossing to A and selective elimination of the segregants containing H-2ª (derived from strain A) by challenging them with a strain A tumor. The negative survivors were always bred further in order to retain the H-2 factor derived from the other, foreign strain, simultaneously with the introduction of more and more strain A genetic background. It cannot be excluded that this procedure may fix more than one histocompatibility factor derived from the foreign strain that was used for the original outcross.

The Possible Nature of Variant Formation

At a recent meeting devoted to "Genetic Approaches to Somatic Cell Variation," Ephrussi gave a lucid discussion of the problems involved (1958). Basing his arguments essentially on the classification of Nanney (1958), he abandoned the conventional "geographical" distinction between nuclear and cytoplasmic phenomena in favor of "truly genetic mechanisms that regulate the maintenance of the structural information," as contrasted to "epigenetic" mechanisms "that regulate the expression of genetic potentialities." Epigenetic mechanisms should in turn be distinguished from immediately reversible phenotypic mechanisms of a more trivial nature.

Individuality of Tumors with Regard to Variant Formation

The previous conclusion (G. Klein and E. Klein, 1958) that different tumors of similar origin and morphology were different with regard to the frequency and type of variant formation was fully confirmed by the more extensive later studies. In addition to methylcholanthrene-induced sarcomas, differences of this type were now also found between estrogen-induced lymphomas and mammary adenocarcinomas. This material is too small as yet to judge whether there are any overall differences between the different tumor types.

Although it often happened that the tendency to give rise to a certain type of variant increased or decreased in the course of serial passage, genuine differences were already present on the first testing when the primary tumor itself was inoculated. A few tumors were nonspecific from the beginning and did not lend themselves to the extraction of variant sublines with well-defined compatibilities by serial selective transfer. A fairly large proportion of tumors were entirely limited to their F_1 hybrid genotype or origin and did not yield detectable variants. Others produced variants in one or both parental types in characteristic frequencies.

Differences between Different Genotypes

For each F_1 hybrid tested, one of the parental strains turned out to be a more frequent site of variant formation than the other. There were some other differences between genotypes with regard to the frequency of nonspecific forms and highly specific forms (G. Klein and E. Klein, 1958). At the time of our previous report it appeared that the maternal strain was usually preferred in variant formation. Testings on reciprocal hybrids were not available, however; and in three of the four crosses studied until that time the parental genotype was the same, namely the original A/Sn strain from which the three other coisogenic-resistant lines had been derived. Several alternative explanations were considered, one of which was the possible existence of self-reproducing particles with isoantigenic activity derived from the mother. This possibility was excluded by more recent experiments, however, which showed that the "favored" strain of variant formation remained the same for a given F_1 genotype even if tumors induced in the reciprocal hybrids were tested (cf. Table 2). Several alternative possibilities remain to be considered, but the difference is probably due to the fact that the isoantigenic barriers separating the tumor from the two parental strains differ in number and/or

can be maintained in their new hosts indefinitely. They do not change back to the original condition when returned to the original genotype. Once established, their growth cannot be prevented by the use of preimmunized hosts. In order to avoid the potential source of error that might arise as a result of secondary enhancement in immunized hosts, we have performed control tests on a representative collection of 19 variants in mice preimmunized seven days before the test inoculation. The variants grew in such animals as well as in those where the interval between the immunizing challenge and the actual testing was longer.

If "variant formation" were due to enhancement, there is no reason why "variants" could not be obtained from homozygous tumors. In the case of heterozygous tumors, enhancement would be equally likely to occur in foreign F_1 combinations, or in homozygous strains other than the parental ones This was not the case, however, as previously discussed. On the other hand, "false positives" could be obtained from homozygous tumors and also from heterozygous tumors in genotypes outside the parental types. They failed to breed true on repeated testing and could be prevented, to a certain extent at least, by the use of preimmunized mice. False positives are more likely to belong to the same category of phenomena as "immunological enhancement."

In order to test whether enhancement by antiserum treatment can facilitate the establishment of "variants" in our system, experiments have been started to test the behavior of F_1 tumors, growing in "enhanced" mice of one of the parental strains. Our experience is limited to two tumor-host combinations as yet. In these cases, growth could be obtained in the parental mice pretreated with antiserum, but the "enhanced" tumors failed to breed true on repeated testing in untreated mice of the same strain and would therefore be classified as "false positives" rather than "variants"

Although variant formation thus appears to be different from immunological enhancement, it does not necessarily follow that antiserum or other influences of the parental host are of no importance for its mechanism. Another case of tumor modification has been described (Barrett and Deringer, 1950; Barrett, Deringer, and Hansen, 1953) that is clearly due to an interaction between some isoantigenic system(s) of a homozygous tumor (probably not the H-2 system) and a heterozygous, compatible F_1 host (G Klein and E. Klein, 1957). This modification is not due to selection and it brings about a permanent specific alteration of the tumor. A parallel has

In a thoughtful paper, Nanney (1958) points out the great practical difficulties involved in distinguishing between genetic and epigenetic mechanisms. Neither persistence nor predictability, neither nuclear nor cytoplasmic localization, can be used with certainty for the classification of a given control system. One criterion that he considers as being of some value lies in the lesser stability of epigenetic systems and their higher susceptibility to extrinsic control.

A tentative discussion on the possible nature of our "variant formation" must remain utterly speculative. The phenomena observed can be considered from various angles. Epigenetic mechanisms could be envisaged if the origin of variants were identical or analogous with certain other known phenomena, essentially due to the inducive action of some factor in the new host environment. In particular, "immunological enhancement" by antiserum (Kaliss, 1958) and the induced adaptation of Barrett and his co-workers (1950, 1953) may be considered. The former phenomenon can be described as the growth of otherwise specific tumors in certain foreign hosts that have been pretreated with lyophilized tissues of the same H-2 constitution as the tumor, or with antisera directed against the H-2 components differentiating the tumor from the host. The presence of the antiserum can be effected "either by active immunization with tissues from the strain of mice to which the test tumor is indigenous or by passive immunization with hetero- or isoantisera" (Kaliss, 1958). The *modus operandi* of immunological enhancement has been pinpointed as the exposure of the tumor graft to a specific humoral antibody. According to one hypothesis, enhancement is due to some "physiological" alteration in the tumor, induced by its contact with antiserum, which insures its survival despite the hostile responses of the host. When enhanced tumors are tested in mice preimmunized by a nonenhanced tumor, they usually fail to grow, at least when the time interval between the immunizing (first) inoculum and the grafting of the enhanced tumor is short enough; e.g., seven days. Enhanced tumors were found to grow for a short number of serial passages in untreated mice of the foreign strain concerned but died out eventually after a variable number of transfers. All published experiments with enhanced tumors deal with the growth of tumors derived from homozygous strains in other homozygotes of foreign strains.

It might be speculated that our "variant formation" could be due to the contact of the tumor cells with an antibody and subsequent immunological enhancement. There are several reasons, however, that make this explanation rather unlikely. Variants "breed true" and

and A SW × A CA F_1). Although both tumors could be regularly recovered from the newborn hosts and were unchanged in their transplantation specificity, we have not obtained any evidence to indicate the occurrence of genetic recombination so far.

We may now consider the arguments that would favor an "immunoselection" (Hauschka, 1957) of isoantigenic cell variants, preexistent in the F_1 tumor prior to testing in the parental strains. The parental environment would have a purely selective effect in this case, in analogy with our model experiments with artificial mixtures (G. Klein and E. Klein, 1956). This explanation has been considered previously and was regarded as fairly probable for the following reasons.

Chromosomal analysis of variants obtained from the MSWB and MACC sarcomas revealed that different variants arising from the same tumor at different times in the same parental strain have an individually characteristic chromosomal constitution that differentiates them from each other and from the original line (Bayreuther and E. Klein, 1958). In one instance, two variants that arose in two different A.SW hosts after the inoculation of two different aliquots of the same pool of tumor cells derived directly from the original line of the MSWB sarcoma (maintained in A × A.SW F_1 hybrids) had exactly the same chromosome constitution. This evidence tends to indicate that we are dealing in this case with the repeated selection of a single variant clone. In another case, it was possible to trace the appearance of a variant compatible with the parental A strain within the original unselected population of the MACC sarcoma because of its deviating chromosomal characteristics. Suggestive as they are, these findings are, nevertheless, only of indirect value since the frequency of variants was not appreciably reduced when very small cell doses were inoculated into the parental strains, thus minimizing the importance of selecting preformed variant clones (G. Klein and E. Klein, 1958; Bayreuther and E Klein, 1958). Also, cases of variant formation have been recently found by K. E. Hellström (personal communication) where a chromosomal change does not seem to accompany the establishment of variant lines.

If it were assumed that variant formation is due to random changes at the genetic level, various mechanisms might be discussed, such as point mutations, more extensive chromosomal rearrangements, somatic segregation, etc. The likelihood of point mutations could be questioned since several isoantigens are usually lost simultaneously, such as D and K, which are now believed to be parts of a

been drawn between this change and certain serotype transformations in ciliate protozoa (Schultz, 1959); the latter is one of the prototypes of epigenetic control systems. The present instance of variant formation is not unlike another observation belonging to the epigenetic category—made on Paramecium by Sonneborn, Schneller, and Craig (1957) concerning the behavior of certain serotype alleles —both of which are expressed in heterozygotes. Loss of the phenotype corresponding to one allele was repeatedly observed. This was not due to total loss of the corresponding allele, however, since the missing phenotype could be restored by change of temperature. The findings were interpreted as being due to differential expression of alleles in heterozygotes. Homozygotes for serotype genes and for other genes did not show this variation. By analogy, it might be speculated that exposure of heterozygous tumor cells to the environment of one parental strain may result in the differential suppression of the isoantigenic products of the H-2 allele derived from the opposite parental type due to the action of an antibody or by other means. Experiments are now in progress to investigate the possibility of restoring the missing antigens by various manipulations *in vitro* and *in vivo*.

Meanwhile, other explanations are by no means excluded. Ambiguities of interpretation are due to the difficulties involved in all attempts to distinguish between genotype and phenotype in somatic cells. Host-induced modifications at the genetic level would include phenomena analogous to transduction involving the incorporation of genetic material from the host, or nonspecific increases in genetic variation provoked by some factor involved in the homograft response of the parental strain. There is no binding evidence available for the occurrence of the latter phenomenon as yet; the former can be excluded because of the lack of variants specific for other F_1 types and containing one H-2 allele foreign to the tumor (Table 4) In the case of transduction this occurrence would be no less likely than the appearance of variants compatible with the parental types. It may be relevant to mention in this connection that we have performed a series of experiments in order to detect the possible occurrence of genetic recombination between two different tumors by mixing two sarcomas of A × A.SW and A.BY × A.CA F_1 hybrid origin, respectively, inoculating the mixtures into "neutral" newborn C3H or DBA mice, and testing the resulting tumors in the two original genotypes as well as in the four different F_1 hosts that would be capable of selecting "recombinant" cells (A × A.BY, A × A.CA, A.SW × A.BY,

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complex pseudoallelic system, and crossing over has been demonstrated between them (Hoecker, 1956; Gorer, 1956). Also, if point mutations were responsible, it is difficult to see why it is impossible to recover some mutants selectively compatible with new F_1 hybrid combinations. More extensive chromosomal rearrangements have been actually demonstrated in connection with some types of variant formation (Bayreuther and E. Klein, 1958). Such rearrangements might involve the H-2 locus or, by changing the genetic background, change the mutability and/or expression of H-2-determined isoantigenic systems. The general validity of chromosomal changes may be questioned, however, on basis of the above-mentioned recent findings of Hellström. Finally, somatic segregation may be assumed as a possible mechanism, but its likelihood can be questioned because of theoretical considerations (probable absence of somatic pairing in cells of this type) and because the frequency of the two complementary types obtained from a given tumor is seldom equal and may show very considerable differences. The latter argument is not entirely conclusive, however, since additional, non-H-2 differences between IR-lines may interfere with the manifestation of one of the two complementary types.

Thus, it must be concluded that while the appearance of specific variants compatible with one of the parental strains seems to be a phenomenon limited to heterozygous F_1 tumors and is distinctly different from the development of "false positives" which grow in spite of a homograft reaction, and from immunological enhancement, the details of the cellular mechanism underlying the change and its genetic or epigenetic nature remain to be investigated.

Experimental Analysis of Tumor Progression

The preceding chapter may serve to illustrate the difficulties encountered in genetic studies on neoplastic cell populations, even if they are carried out with marker characteristics determined by known genetic mechanisms and selectively detectable in appropriate experimental systems. Even greater difficulties may be envisaged with less clear-cut and less selective systems. As, we hope, the experiments discussed have shown, a certain amount of information could be easily obtained with the H-2 system; variants of different types could be extracted and characterized, and the frequency of variant formation should be amenable to quantitative study. The difficulties are encountered when trying to interpret the findings in terms of cellular mechanisms; the same is true for many other non-Mendelian sys-

tems (Nanney, 1958). Apart from these difficulties, however, there are some striking parallels between the empirical "rules" of progression (Foulds, 1954, 1958) and the observed characteristics of variant formation Progression occurs independently in different tumors and leads to an independent reassortment of different characteristics—different tumors show an individuality with regard to the occurrence and frequency of given variants. Different unit characters undergo progression independently from each other—different isoantigenic variants can be obtained from the same tumor. Progression may occur in distinct steps—so can variant formation. Both progression and variant formation can follow one of alternative paths of development. Progression is essentially a one-way process—the permanence and stability of variant formation is equally irreversible. Variant formation represents the loss of certain isoantigens, the possession of which interferes with growth in a given foreign genotype—the various changes involved in progression may be viewed as representing the gradual loss of cellular responsiveness to the superimposed, inhibitory influences of various homeostatic control forces.

Can we assume that variant formation is essentially the same phenomenon as the cellular changes underlying progression? We would hesitate to do so Since the competence of a differentiated mammalian cell to respond to the growth stimulating and/or inhibiting influences of superimposed homeostatic control mechanisms must be organized in a subtle and complicated way, there is no reason why such a system would not be vulnerable at many different points and why different forms of cellular change could not participate in facilitating the development of the neoplastic cell population toward ever increasing independence. In the words of Lederberg (1958): "Present knowledge places no bounds on the scope of mechanisms of variation that might contribute to a neoplastic phenotype, just as we do not attempt to account for the evolution of species by any single mode of genetic displacement. Indeed, it would be surprising if a neoplastic phenotype were always initiated by a single variational event, and we can suppose that the cumulation of several variations (whether by gene mutation, virus infection and transduction, recombination, or karyotypic upsets) will be necessary before a once normal clone transcends the threshold of malignancy."

Presently available experimental designs permit certain types of studies at the population level and at the cellular level that may be informative, not with regard to the origin of the cancer cell, but in characterizing sharp and well-defined biological changes of one or

several of the "unit characteristics" related to malignancy. At the population level, one may investigate whether a given change is due to the selection of randomly occurring variants or to the inducing action of some environmental factor. In order to gain information about the changes at the cellular level, one may carry out comparisons between different sublines of the same original neoplastic cell population that differ, either because of a spontaneous change or as a result of experimental selection, with regard to one or a few "unit characteristics," such as hormone dependence, metastasizability, invasiveness, drug sensitivity, etc. Such comparisons, whether by cytological or histological examination, or by biochemical, biophysical, or immunological methods, may give better information about the structural correlates of the biological change than more conventional comparisons between malignant tissues and their "homologous" normal counterparts that may differ in many other, less relevant respects. Examination of the details of chromosome morphology concurrent with such changes may present sharper systems for the assessment of the possible role that chromosomal modifications may play in tumor development than what is available now in the form of more general studies on various normal and malignant tissues from the chromosomal point of view.

Some studies of this type have been carried out already in a more or less preliminary fashion. They may serve to illustrate the manifoldness of the neoplastic evolution and the improbability of general unified concepts. Changes based on intercellular variation followed by environmental selection have been documented in the cases of drug resistance in mouse leukemia (Law, 1952), development of changed transplantation characteristics together with chromosomal rearrangements (Hauschka, 1957, 1958), appearance of variant sarcoma cells capable of growing in the dissociated free cell form of an ascites tumor in a mouse sarcoma previously inconvertible to the ascites form (E. Klein, 1955, G. Klein and E. Klein, 1955). However, as mentioned previously, a host-induced modification of an isoantigenic system (Barrett and Deringer, 1950; Barrett, Deringer, and Hansen, 1953) has been shown not to depend on selection but on some more direct environmental effect on the tumor cells (G. Klein and E. Klein, 1957). Still other mechanisms may exist at the population level, involving self-stimulation of the tumor cells after a certain critical threshold of colony size has been reached, with an automatic increase in autonomy (*loc. cit.*).

Comparisons between parallel cell lines selected from the same

original cell population and differing in one or a few unit characteristics have been limited to the study of sarcoma sublines differing in ascites convertibility, metastasizability, invasiveness, and adhesiveness (Ringertz, E. Klein, and G. Klein, 1957; Purdom, Ambrose, and G. Klein, 1958) and to tumors that are sensitive and resistant, respectively, to certain antimetabolites or other growth inhibitors (Law, 1956; Potter, 1958). The former comparison indicated a difference in the structure of the cell surface, manifested in differences of the negative electrical charge (Purdom, Ambrose, and G. Klein, 1958), while the latter type of study suggests that the development of resistance toward a given carcinostatic agent may proceed along different pathways in the same type of tumor cell.

Although studies of the type suggested may appear cumbersome in technique and limited in scope, and may often lead to negative or ambiguous results, nothing less than a thorough factorial analysis seems to be capable of resolving the multifaceted complex of phenomena grouped together under the nosological concept of malignancy.

Summary

Biological studies demonstrating the gradual development of certain tumors by a series of qualitative, permanent, and irreversible cellular changes ("tumor progression") indicate (Foulds, 1954) that an independent reassortment of various "unit characteristics" is taking place before the appearance of the final neoplastic condition. The unit characteristics in question, such as hormone dependence, invasiveness, metastasizability, growth rate, degree of differentiation, loss of secretory functions, etc., are all parts of the complex phenomenon of malignancy, but their unpredictable and random change during neoplastic development and the unique and individual picture that results from their independent reassortment in every single tumor suggest that the underlying cellular changes are not necessarily related to each other. It would follow that the neoplastic microevolution may proceed along various alternative pathways.

The genetic analysis of tumor progression—reminiscent of the adaptive evolution of microorganismal populations—is hampered by the absence of direct methods of genetic transfer between somatic cells and the impossibility of distinguishing between genotype and phenotype. Indirect methods of various kinds have been used in recent years and a certain amount of circumstantial evidence has been collected. Genetically determined marker characteristics, detectable

at the cellular level by selective techniques, are now becoming increasingly available. The isoantigenic markers determined by the system of histocompatibility genes in the mouse have been studied most extensively.

As a model system for analyzing isoantigenic variation, we have been particularly interested in tumors induced in F_1 hybrid mice, produced by crossing two of the isogenic resistant lines of Snell (1948, 1958b). Such hybrids are heterozygous with regard to the histocompatibility-2 (*H-2*) *locus* while the rest of the genome is theoretically homozygous. H-2 is a good marker since its products are isoantigenically active and are detectable, under suitable conditions, at the cellular level by selective techniques, involving serological and transplantation tests. By methods of selective transfer it was possible to establish variant sublines from several F_1 tumors, compatible with one or the other of the parental strains and characterized by the loss of specific H-2-determined isoantigens. No such variants could be obtained from tumors of homozygous origin. With the heterozygotes, no specific variants could be obtained that would have required the gain of isoantigen(s) concurrently with the loss of an alternative one. "Recombination" experiments involving mixed growth of two tumors with different markers gave negative results.

A closer analysis revealed that variant formation can proceed in several distinct steps and different types of variants can be obtained from the same F_1 tumor. Characteristics of a given variant remain constant even after return to and prolonged passage in the original F_1 genotype. Different tumors of similar origin and histology are individually different with regard to variant formation. This may be related to the fact, shown by cytological evidence on the same material (Bayreuther and E Klein, 1958), that an individual remodeling of the chromosome set has occurred in every single tumor. Similar findings have been reported by Ford, Hamerton, and Mole (1958) on other types of neoplastic tissues. Although it is uncertain whether the isoantigenic variation is directly related to variations at the genetic level and epigenetic control systems might well be involved, the permanence of the changes and their relative insensitivity to extrinsic influences make a genetic basis more probable than other alternative hypotheses.

Isoantigenic variant formation has several features in common with the process of tumor progression. The former can be viewed as the loss of isoantigens preventing growth in a foreign-host genotype while the latter is essentially a gradual loss of responsiveness to vari-

ous growth-controlling homeostatic forces in the autochthonous host. In spite of this and other similarities, isoantigenic variant formation must be regarded as a model of only some cases of progression at its best. There is some evidence to indicate that progression may proceed by different mechanisms at the population level. Several cases of variation selection, one instance of a host-induced adaptive modification, and an automatic change in responsiveness due to increase in population size have been described. Only a thorough factorial analysis of clear-cut unit changes isolated from the tremendous complexity of tumor progression can be hoped to clarify the various cellular mechanisms involved in the development of malignancy.

ACKNOWLEDGMENTS

This work has been supported by research grants from the Swedish Cancer Society and by Grant C-3700 from the National Cancer Institute, U S Public Health Service Our thanks are due to Miss Barbro Lindstrom and Miss Maj-Lis Eriksson for their skillful and dependable technical assistance

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Sex-Linked Incompatibility of Male Skin and Primary Tumors Transplanted to Isologous Female Mice

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The isoantigens of mammalian cells, both normal and malignant, are controlled by dominant histocompatibility genes. In the mouse, these H factors are distributed over several chromosomes. They produce either strong or weak antigenic substances without apparent interaction among themselves. In order to accept a graft permanently, the recipient must match all the antigens present in the transplant. Antigens peculiar to the host, but lacking in the graft, are immaterial for compatibility.

These genetic principles of tissue immunity have emerged largely from experiments with transplantable mouse tumors because the antigens determining regression of these tumors are relatively few (see current reviews by Gorer, 1956; Hoecker, 1956; Amos, in press).

The more complex antigenic prerequisites for the survival of normal tissues have handicapped their analysis. For example, skin from strain A takes in (A × CBA)F_1 hybrids only if an estimated 15 antigens coincide in such a mouse; two takes among 120 recipients of skin grafts were recorded by Barnes and Krohn (1957). The laboriously developed isogenic resistant line pairs of Snell, differing at a known histocompatibility locus, have partly overcome these experimental obstacles (Counce, Smith, Barth, and Snell, 1956).

Intrastrain grafts were believed a priori to be 100 per cent com-

patible, hence were of no genetic interest until Eichwald and Silmser (1955) discovered a sex influence on graft survival within certain strains. Female mice frequently reject isologous skin grafts from males; whereas transplants from female to female, female to male, and male to male are accepted. An inbred strain in which the sex-linked incompatibility is consistent (though weak, as compared with H-2 effects) would furnish ideal isogenic antibody producers for refined serologic investigation.

The original data of Eichwald and Silmser were interpreted at their request by Hauschka (1955), who proposed Y-linkage of a histocompatibility gene as the most likely explanation of the challenging results. Endocrine requirements of male skin lacking in the female host were considered a less probable alternative. Snell (1956) found Y-linkage "both intriguing and plausible," but doubted whether firm proof could ever be obtained.

One recent genetic test for Y-linkage (Bernstein, Silvers, and Silvers, 1958) was inconclusive since both parental strains used in the test cross carried the "male factor." Meanwhile, Eichwald, Silmser, and Weissman (1958) have virtually eliminated a hormonal mechanism. Castration, testosterone treatment, or implants of testes did not abrogate female incompatibility toward male skin.

Our own experiments include over 400 skin grafts in several inbred strains and in F_1 hybrids. Male tolerance was induced in refractory females by several methods, and we performed serological tests with isologous anti-male sera from hyperimmune C57BL females. The presence and stability of the "male antigen" were examined in five neoplasms.

Sex-Linked Isolation of an Antigen in the Male Genome of Some Inbred Strains and Antigenic "Contamination" of the Females in Other Strains

Methods

Our skin grafts were made following the general recommendations of Billingham and Medawar (1951). Attention was paid to the periodicity of hair growth. We avoided grafting skin in the unfavorable stages of "anagen" during which the epidermis, the dermis, and the adipose layer become increasingly thicker (Montagna, 1956).

Donor and recipient were anesthetized with Sodium Nembutal. On both mice, areas considerably larger than the middorsal operative field were shaved and then swabbed with a 1 : 1000 aqueous solu-

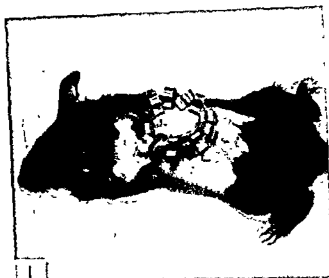




Figure 1 Anesthetized $C3H_f/Ha$ mouse immediately after skin isograft was fastened with a ring of Michel wound clips

Figure 2 $C3H_f/Ha$ agouti female (genotype *Pp*) bearing a four-month-old successful isograft from a pink-eyed dilute agouti male (*pp*).

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tion of Zephiran chloride (Winthrop Stearns, Inc.). A full thickness pinch-graft, about 1.5 to 2.0 cm. in diameter, was cut from the donor, put into ice-cold sterile Earle solution in a Petri dish, and dissected free of vessels, subcutaneous fat, and muscle. Bleeding from the panniculus carnosus was avoided as much as possible while a graft bed of proper size was opened in the recipient. Unless distinguishable by a different coat color, the graft was fitted to the bed in such fashion that future hair would grow at a right angle to the host's fur. Instead of using sutures (Liebelt, 1957) or gauze dressings, we attached the graft by means of a dozen or more 11 mm. Michel wound clips, which were pinched in place with Michel clip forceps. A ring of these clips surrounding the transplanted skin (Fig. 1) discourages nibbling and facilitates postoperative inspection of graft behavior. Following

TABLE 1. Sex Influence on Intra-strain Rejection of Mouse Skin Grafts

	Successful Takes/Total Grafted				Per Cent Takes
Strain of Donor and Recipient	♀ to ♀	♂ to ♂	♀ to ♂	♂ to ♀	♂ to ♀
C57BL/Ha intrastrain	6/6	6/6	10/10	0/19	0%
A129/Ha intrastrain and 129/Rr to A129/Ha	2/2	8/8	6/6	0/11	0%
Y/HeHa intrastrain	29/33	17/17	24/24	20/34	59%
C3Hf/Ha intrastrain	10/10	2/2	9/9	26/27	96%
DBA/2 intrastrain	3/3	22/22	6/6	8/8	100%

surgery the mice were kept isolated in individual cages. The clips were removed under light ether anesthesia when the graft was one week old. Because of the good anchorage provided by these clips, technical failures were exceedingly rare.

The criteria for take or rejection were: discoloration of the graft, stiffness, desquamation and contraction, growth of graft hair often identifiable by coat color markers, or eventual sloughing, scabbing, and balding. Grafts were inspected at brief intervals for an average observation period of 27 weeks before they were classified. In a few instances of possible technical failure, second grafts of appropriate genotype were made in order to double-check the host response.

Very advantageous for the skin graft work were special inbred

strains, or mutant sublines thereof, segregating for a single coat color difference on an otherwise isogenic background. Availability of these color markers at a 1:1 ratio was controlled by the following sib-mating schemes:

Y/HeHa—yellow ($A^y a, bb$) × brown (aa, bb)
DBA2/Ha—Maltesian dilute (dd) × intense (Dd)
$C3H_t$/Ha—pink-eyed dilute (pp) × intense (Pp)
A129/Ha—off-white (c, ch) × chinchilla (ch, ch)

A129/Ha is isogenic with 129RrJax, except for the albino locus.

Whenever possible we made use of the above color "markers" for convenient recognition of intrastrain skin grafts. None of these coat color differences interfered with the histocompatibility of skin

Results

Table 1 summarizes the results for 273 skin grafts within five inbred strains of mice. The first three sex combinations (♀ → ♀, ♂ → ♂, ♀ → ♂) of donor and recipient were usually compatible.

However, the reaction of females toward male transplants differed from strain to strain. C57BL/Ha and A129/Ha females always rejected male skin. The yellow Y/HeHa strain gave intermediate results. Among 27 $C3H_t$/Ha females only one was incompatible, while all DBA/2 females were tolerant. Sloughing symptoms of male skin in the C57BL females first became apparent at 13.9 ± 1.3 days, in the A129 at 15.6 ± 0.9 days, and in the yellow mice at 35.3 ± 11.0

TABLE 2 Behavior of Mouse Skin Grafts in Hybrids between the C57BL/Ha and $C3H_t$/Ha Strains

Genotype of Donor and Recipient*	Successful Takes/Total Grafted ♂ to ♂	♀ to ♂	♂ to ♀	Per Cent Takes ♂ to ♀
C57BL to (C3H × C57BL) or reciprocal F_1			23/28	82%
(C3H × C57BL) to same F_1		11/11	3/11	27%
(C57BL × C3H) to same F_1		11/11	16/20	80%
(C3H × C57BL) to reciprocal F_1	7/7			
(C57BL × C3H) to reciprocal F_1	7/7			

* The first named strain between parentheses is always the female parent (♀ × ♂).

TABLE 3. Progressive Change and Subline Difference in the Compatibility of Female Y/HeHa Mice for Male Skin

Y/HeHa Subline or *Hybrid* Combination	Date Grafted	Successful Takes/Total Grafted			
		♀ to ♀	♂ to ♂	♀ to ♂	♂ to ♀
Intrastrain, Y Subline unknown	Feb.–June '57	3/5	5/5	13/13	1/8 (12%
(C57BL × Y) to same F_1, Y Subline unknown	Aug. '57	.	.	6/6	0/5 (0%
Within Subline 1*	Oct. '57–Jan. '59	14/16	4/4	2/2	7/14 (50%
Within Subline 2*	Oct. '57–Jan. '59		2/2	7/7	11/11 (100%
Subline 1 to 2	Nov. '58–Jan. '59	5/5	6/6	.	1/1
Subline 2 to 1	Nov. '58–Jan. '59	7/7		2/2	.

* Sublines 1 and 2 were derived at our laboratory from the same litter in November, 195 They have been carried as two separate sib-mated lines since then.

days. A few second grafts began to deteriorate in less than half this time.

The finding that our $C3H_t$/Ha females were nearly always compatible with male tissue suggested two possibilities: (1) The C3H *male factor* was weaker than that in the C57BL mice; and (2) the C3H autosomes or X chromosomes had become "contaminated" with the Y chromosome region producing the male antigen, and sex linkage was thereby obscured.

The hybrid data presented in Table 2 support the latter alternative. *Compatibility with the male antigen can be transmitted either by* C3H females or males (Table 2, line 1) into F_1 daughters from an outcross to C57BL. Transmission of compatibility is interpreted as transmission of the antigen itself.

It appears certain from intrastrain grafts in C57BL that the X *chromosomes and autosomes of this strain were* "uncontaminated" by the Y-linked male antigen. It is further apparent that not all C3H mice transmitted the male factor. Skin grafts between reciprocal F_1 males were all successful. They indicate that C3H males transmit the factor to their F_1 sons on the Y chromosome and, further, that the Y *factor* in C3H and C57BL males is the same.

Male C57BL/Ha or F_1 skin contained an antigen strong enough to elicit a typical sloughing response within 20 days, and an accel-

erated "second set" reaction in a few F_1 females; yet identical male grafts were permanently accepted by the majority of similar F_1 females. This lack of response, however, need not be attributed to subthreshold amounts of a weak male antigen (Snell, 1956; Eichwald, Silmser and Wheeler, 1957). Our data permit the alternative assumption that compatible F_1 females in Table 2 and in the $C3H_f$/Ha strain cannot react against an antigen which they themselves produce, or to which they are tolerant.

The results with the yellow mice (Table 3) lend further support to this view. A progressive change was observed in the frequency with which male grafts were accepted by Y/HeHa females. To emphasize this change the data are arranged in time sequence.

Before October 1957, mice in both of our essentially coisogenic sublines of the yellow strain were grafted, within each subline but without written record of the subline, when they had reached the age of about two months. During this early stage of the work, male Y/HeHa or (C57 × Y)F_1 hybrid grafts were nearly always rejected by females.

In a later series, only half (7 of 14) of the male-to-female grafts within subline 1 were sloughed; it is noteworthy that five among the seven takes have occurred during the last six months. Does this signify progressive "contamination" of the subline 1 females by the male factor? Meanwhile, subline 2 females have become entirely compatible with male tissue One such individual now bears three successive male skin grafts, all in excellent condition. This cannot be explained away by simply assuming a weak male factor, for even a weak antigen would surely have elicited a detectable immune response under this wholesale onslaught.

From a theoretical point of view, the four rejections of female skin by females, recorded in Table 3, column 3, are important. These four female grafts were sloughed within 30 days and behaved as if they were male. They cannot be dismissed as technical failures. All four rejections occurred at a time when the male-incompatible females (*MI*) in the yellow stock still greatly outnumbered the male-compatible (*MC*) females. All of the latter presumably have acquired the male antigen (see "Discussion"). It is postulated that *MC* female skin is rejected by *MI*, but not by *MC* females, while *MI* female skin is compatible with both types of females. Unfortunately, this proposal no longer can be tested since all our yellow females now appear to be *MC*, as is evident from the last two lines of column 3, Table 3

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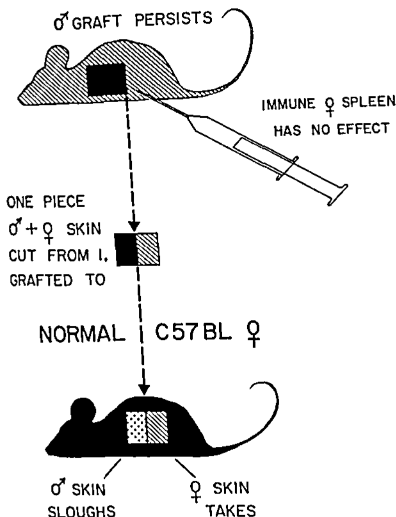


Figure 3 Experimental scheme for grafting skin from nonirradiated C57BL males to lethally irradiated, marrow-protected females, where graft persists indefinitely and is unresponsive to immune lymph nodes and spleen Upon regrafting six months later to a second normal C57BL female, together with a piece of grayed female skin, the male portion of this dual graft is rejected, while the female skin is accepted

Induced Tolerance to Male Skin in Refractory Females

Main and Prehn (1955) reported prolonged survival of skin homografts between inbred strains of mice after a lethal x-ray dose to the recipients which then were protected with homologous bone marrow.

Utilizing this technique, we gave 800r total body irradiation[1] to eight C57BL/Ha females, and injected them intravenously with isologous marrow; five of these mice received male marrow, three were given female marrow, and all survived. One month after irradiation they were grafted with normal male C57BL skin which took equally well whether the protective marrow had been male or female.

In two other groups treated with 800r under partial shielding, or with only 300r x-irradiation, five out of 16 C57BL females retained their ability to reject male grafts. Sufficient numbers of immunologically responsive host cells appear to have survived in these five mice.

TABLE 4 Experimentally Induced Tolerance to Male C57BL Skin in C57BL Females

Pretreatment of ♀ Recipients	♂ Skin Takes/Total ♀♀
Untreated controls	0/19
X-ray 800r total body, then C57BL marrow i v.	8/8
X-ray 800r, but one hind leg shielded	9/12
X-ray 300r, no shielding	2/4
i p. injection of ♂ C57BL spleen at age <12 hours	2/2
Foster nursing on C3H$_f$/HA ♀ carrier of ♂ antigen	1/3
Foster nursing on DBA/2 ♀	0/5

The 19 male grafts that were accepted by the irradiated females (see Table 4) grew jet-black hair distinguishable from the graying coats of their hosts, and persisted in excellent condition. We attempted, without success, to cause breakdown of one such male graft by injecting minced spleen and lymph nodes from a hyperimmune C57BL female which had sloughed two successive male grafts.

This failure called for a test which would show whether the male grafts surviving for half a year on their irradiated hosts had perhaps

[1] 250 KVP, 0.25 mm. Cu + 1.0 mm Al, HVL 0.95 mm. Cu

THE SEROLOGICAL BASIS OF FEMALE INCOMPATIBILITY FOR MALE SKIN, AND ABSENCE OF AN IMMUNOLOGICAL INFLUENCE ON SEX RATIO

All the data so far presented are compatible with the concept (Hauschka, 1955) that the basis for the rejection of a male graft by an isogenic female is a Y-linked antigenic difference between the two. The hypothetical antigen would evoke an immune response in any animal not carrying Y or the effective region of Y.

Presumptive support for the primary role of an immunological mechanism in this sex-linked graft rejection comes from accelerated sloughing, the "second set" response to subsequent skin grafts after the first has failed (Eichwald, Silmser, and Weissman, 1958; and data presented here).

More rapid rejection also has been induced by pretreating female mice with male tissue mince prior to grafting male skin on these preimmunized hosts (Sachs and Heller, 1958). Billingham and Silvers (1958) induced tolerance to the hypothetical male antigen by injecting male spleen cells into newborn C57BL females. This treatment abolished their ability to reject male skin as adults The only published attempt to demonstrate serologically the antibodies in this sex-linked system is that of Sachs and Heller (1958). Their test for the presence of hemagglutinins after male-to-female immunization was, however, negative.

We obtained our anti-male sera from hyperimmune C57BL females that had rejected two or three consecutive male skin grafts and examined them for hemagglutinins, leuko-agglutinins, and leukocytotoxins.

No hemagglutinins were found whether the tests with C57BL male red cells were carried out in saline or in a dextran:human serum-enhanced system (see Gorer, 1956). Leuko-agglutination was carried out using a washed suspension of cells from perfused C57BL spleens, serum and cells were diluted in saline. Six individual sera have so far been tested; four gave positive results with male spleen as compared to female spleen that was included as a control. Serum from normal C57BL males or females, or from a female that had accepted a female skin graft, was negative The white cell agglutination was weak compared to the strong reactions one is accustomed to seeing with H-2 antisera, but was considerably stronger than any previously obtained in other systems with anti-H-1 or H-3. Confir-

lost the male antigen. Five such male grafts were removed and regrafted to normal C57BL females. These were promptly rejected. Four dual grafts consisting of male plus irradiated female skin were transferred to normal recipients following the scheme in Figure 3. The black male half of these rectangular grafts perished within four weeks, while the female skin persisted and grew white hair. Apparently, the male antigen in skin is not lost or weakened during long residence in a tolerant female.

Billingham and Silvers (1958) and Mariani, Martinez, Smith, and Good (1958) induced tolerance to male skin in C57BL and A females which had been injected intravenously as newborn infants with adult male spleen. We repeated this experiment on a smaller scale as shown in Table 4, line 5, and also obtained two takes of 129/Rr *male skin in two C57BL females injected at birth with corresponding* male spleen. The results give further support to a straightforward immunogenetic interpretation of female incompatibility for male isografts.

There is yet another possible source of female tolerance which may be operative in nature; namely, the maternal uterine and/or milk influence (Barrett and Morgan, 1949). Peer (1957) has obtained much longer survival of human skin homografts from mother to child than from father to child. Fetal exposure to maternal antigens was held responsible for this difference, which was not related to sex or age of the child.

Foster nursing is capable of inducing tolerance to leukemic and other tumor transplants in normally resistant mice (Cloudman, 1941; Law, 1942). The last two lines of Table 4 show preliminary results from a larger experiment now in progress. One of three C57BL/Ha females, fostered from the day of birth to weaning age by a $C3H_f$/Ha female, *later accepted a male C57BL skin graft. In our black strain,* no untreated female had ever done this. The foster mother was compatible with an isologous male graft of long standing. Since the male antigen in our C3H and C57BL mice is the same (see Table 2) and is presumably present in male-compatible C3H females, it may be transmissible by the milk in solution or on maternal cells. The milk influence could thus be interpreted as "actively acquired tolerance" in the sense of Billingham, Brent, and Medawar (1953). However, the small amount of antigen transferred in this manner rarely may be enough for lasting tolerance to skin, although sufficient for the brief progressive growth of a neoplasm.

The Serological Basis of Female Incompatibility for Male Skin, and Absence of an Immunological Influence on Sex Ratio

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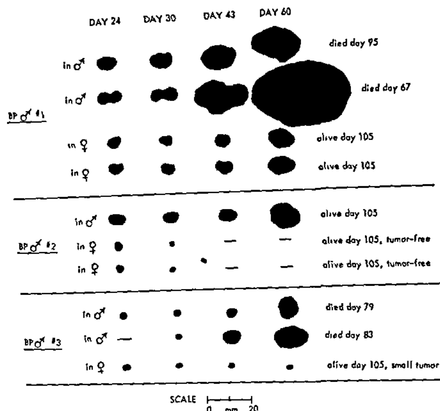


Figure 4

equally well in mice of both sexes during the first two serial passages. After six weeks of growth, mean tumor diameter of BP ♀ #4 was 22.0 ± 2.2 mm in females, and 25.0 ± 2.2 mm. in corresponding males. Since there also was no significant difference in survival time, measurements on this material were discontinued.

The most rapidly growing male sarcoma was BP ♂ #1. Its "progression," therefore, was observed through two further male and female passages. In both instances, tumor tissue was taken from a male of the preceding transfer for transplantation into both sexes. This was done in order to avoid cumulative damage to the sarcoma cells resulting from a possible immune response during their continu-

mation was afforded by a cytotoxicity test (method of Gorer and O'Gorman, 1956) in which the C57BL spleen cells were incubated with the antiserum in the presence of normal guinea pig serum and 1:2000 trypan blue as an indicator of cell damage. About 6 per cent staining of male cells was consistently obtained with serum from the immune females as compared with control values of 1 to 2 per cent. There was no increase in staining with female cells. This was in keeping with the leuko-agglutination results and indicated the presence of a weak but definite cytotoxin.

Feldman (1958) and Sachs and Heller (1958) have speculated regarding experimental sex control by selective cytotoxic effects of female antisera on sperm carrying the Y chromosome. With this possibility in mind we mated five hyperimmune C57BL females which had rejected two successive isologous male skin grafts to normal C57BL males and recorded sex ratios at birth. The sex distribution ratio for 26 litters, totaling 141 young, was 49.0 ± 4.2 per cent male. This is not significantly different from the sex-ratio at birth of 50.8 ± 1.2 per cent calculated for 293 litters of our normal C57BL breeders. *In utero,* at least, male incompatibility does not operate.

Instability of the Male Antigen in Tumors

Transplantable mouse tumors often become heteroploid during serial passage (Hauschka, 1957), and this may be accompanied by loss of strain specificity and diminished absorbing titers for H-2 antisera (Hauschka and Amos, 1957). It was, therefore, desirable for the present purpose to work with primary tumors, which usually have near-diploid chromosome constitutions. The preferred host strain was C57BL/Ha because females of this stock had consistently rejected male skin grafts.

Sarcomas BP ♂ #1,2,3 and BP ♀ #4,5,6 were induced in C57BL mice by subpannicular injection of benzo [a] pyrene. The resulting growths were excised from their primary hosts when they were still small and firm, in order to exclude variables introduced by necrotic tissue, and were cut into uniform cubes fitting snugly into a 13-gauge trocar. The tumors then were transplanted into the chest walls of a few isologous males and females, eight to ten weeks old. The small amount of primary tumor tissue available limited the size of the experiment. Nevertheless, the three sets of caliper measurements depicted in Figure 4 demonstrate growth inhibition or complete regression (BP ♂ #2) of the three male sarcomas in C57BL females.

However, the three female sarcomas BP ♀ #4,5, and 6 grew

was still present and could operate in such aneuploid cells by eliciting an inhibitory host response in the female line; and, if so, whether the chromosome constitution of the female subline would show a downward shift under the pressure of immunoselection against the Y chromosome. Since the hypotetraploid EL24 idiogram may be presumed to have no more than one or two Y chromosomes, very slight numerical changes would be anticipated. Two X chromosomes were seen in some metaphase plates, but identification of the Y is still uncertain (Figs. 5 and 6).

A selective shift is suggested by the exact metaphase chromosome counts presented in Table 5. The modal number in the female subline remains at 76, but this peak contains fewer cells than the corresponding male category.

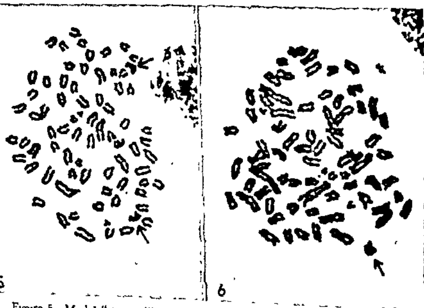


Figure 5 Modal "stem cell" type of the EL24 male ascites lymphoma from C57BL male passage; 76 metaphase chromosomes, including an ever present "minute," may be counted The two, more deeply staining X chromosomes are indicated by arrows Fixation by acetic orcein

Figure 6 EL24 ascites lymphoma cell from the first passage in female C57BL mice. This pro-metaphase plate has 78 chromosomes. The "minute" lies below center, the two heterochromatic, deeply stained X chromosomes are juxtaposed in characteristic somatic pairing (arrow). Fixation by acetic orcein.

ous growth in female passage. Our objective was to learn whether the general syndrome of "tumor progression," unaided by immunoselection (Hauschka, Kvedar, Grinnell, and Amos, 1956), would include early disappearance of the male antigen.

During its second passage, tumor BP ♂ #1 grew at nearly twice the rate shown for the males in Figure 4, but was still somewhat retarded in the females. For the third passage, 46 mice were distributed into the following four groups:

Group I—Eight untreated males;
Group II—Eight males injected intraperitoneally with 91×10^6 nucleated male spleen cells from a pool of minced, screened C57BL spleens. This inoculum was suspended in 0.5 ml. Earle solution and is equivalent to an average-sized whole spleen;
Group III—15 untreated females;
Group IV—15 females injected exactly like Group II with an *immunizing dose of isologous male spleen cells.*

One week after the spleen injection, the mice in the above four groups received implants of second passage BP ♂ #1 from the same individual male donor. However, neither sex nor "preimmunization" exerted a significant influence on tumor development or size. The mean tumor diameters 16 days after implantation were: Group I, 10.4 ± 1.2 mm.; Group II, 8.4 ± 1.3 mm.; Group III, 8.7 ± 0.8 mm., and Group IV, 10.5 ± 1.1 mm. Apparently, sarcoma BP ♂ #1 had already lost its effective male antigen during the second passage in males and was now able to grow at maximum rate even in preimmunized females.

From the following observations on two male ascites lymphomas (EL24 and C58#4) it appears, however, that the male antigen occasionally may persist over longer periods.

Lymphoma EL24 originated in a C57BL male which Dr. F. Bock of this Institute had skin-painted with dimethylbenzanthracene. The primary host contained an ascitic exudate rich in malignant lymphocytes and well suited for exact chromosome counts. The lymphoma had a near diploid nuclear constitution with a secondary mode of about 20 per cent near-tetraploid cells

After 53 serial passages of EL24 in C57BL males, the cell population was entirely hypotetraploid with a sharp mode at 76 chromosomes. At this time we established a female subline. A question of considerable interest was whether the supposedly Y-linked male antigen

TABLE 6 Takes of Male-to-Female Skin Grafts Reported for Various Strains

Inbred Strain of *Mice*	Bernstein et al., '58	Eichwald et al., '58	Hauschka et al., this paper	Mariani et al., '58	Prehn and Main, '56	Short and Sobey, '57	O. Zaalberg '59, in press	Sachs and Heller, '58
C57BL	1/28	0/46	0/19	3/31	0/6	6/37	0/29	0/16
A129/Ha	-	-	0/11	-	-	-	-	-
Y/HeHa	-	-	20/34	-	-	-	-	0/21
C3H	-	1/7	26/27	10/10	7/8	-	-	0/5
DBA/2	5/12	-	8/8	-	9/9	23/25	-	0/4
Balb/c	-	4/49	-	7/10	11/12	-	-	-
CBA	-	-	-	-	-	15/26	27/27	-
ST	-	4/12	-	-	-	-	-	0/21
BRS	-	-	-	-	-	-	-	0/12
A/Jax	-	16/39	-	11/46	-	3/30	-	-
A^W/*Fa*	-	-	-	-	-	32/36	-	-
Ce	-	-	-	4/4	-	-	-	-

TABLE 5. Chromosome Numbers of a C57BL ♂ Tumor in ♂ and ♀ Passage

Lymphoma EL24 Transfer Generation	Chromosome Number														
	67	69	71	72	73	74	75	76 MODE	77	78	79	80	81	82	Total Counts
♂G53 1 mouse*			1	1	3	2	2	17 43%	3	4	3	1	1	1	39
♀G1, G2 4 mice	2	5	2	14	6	8	28	47 32%	17	8	5	3	2		147

* This ♂ furnished the tumor for the ♀ subline.

More convincing is the overall downward trend of chromosome counts obtained from the ascites during the first two passages of EL24 in females; 45 per cent of all cell counts were below 76. In the donor male, only 24 per cent of the metaphases had fewer than 76 chromosomes, and there was no indication of a secondary population component. In female passage, two new minor chromosome classes appeared at 75 and 72.

A probably related phenomenon was the occurrence in females of two successive growth waves which were not seen in males. The females showed marked abdominal distension; the ascites then appeared to regress, abdominal contour returning to almost normal girth for a week, whereupon a second large accumulation of ascites terminated in death of 14 females 33.2 ± 1.4 days after inoculation of 40×10^6 tumor cells. In 21 males the ascites grew progressively, and these males survived for only 22.0 ± 1.0 days. The difference in survival time (11.2 ± 1.73 days) is highly significant.

Confirmatory results were obtained with another ascites tumor, C58#4, which had arisen as a spontaneous thymoma in a C58/McDHa male. This thymoma was carried in males for 30 passages, whereupon total cell number per mouse in the male line was compared with counts in a female subline. The total cell number of ascites in the females showed a lag behind the number in corresponding males; the difference, after four transfers, had become 16-fold.

Discussion

Sex-linked influences on the histocompatibility of transplantable tumors have been observed in earlier investigations (Strong, 1929,

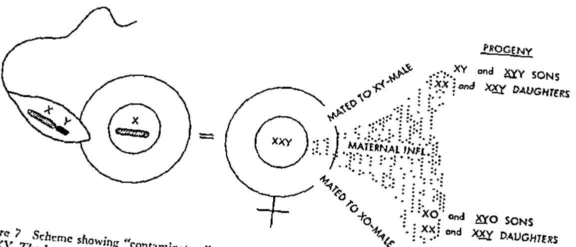


Figure 7 Scheme showing "contamination" of the female *genotype with the male* antigen by a sperm-carrying XY. The latter may represent complete *nondisjunction*, or translocation of part of the Y. Progeny types are shown for a mating of the resulting XXY *female to a normal* XY male, or to a hypothetical, fertile XO male.* Intra-uterine and/or milk (?) *influences also* may induce tolerance to the male antigen in the offspring that lack the Y or active *portion thereof* altogether. A similar scheme would apply if Y *material* were translocated to an autosome.

* Since this work *was submitted to press,* W. J. Welshons and L. B Russell (*Proc. Nat. Acad Sc., U.S.A.*, 95·560–566 [1959]) *have published* genetic and cytologic evidence for the existence of XO *mice* which are, however, females.

Bittner, 1932; Hauschka, Amos, and Grinnell, 1956). But these effects were superimposed on the segregation of autosomal antigenic loci and were beyond the reach of a serologic approach. The sex-linked rejection of isologous tissue discovered by Eichwald and Silmser (1955) has aroused much investigative curiosity because it holds promise for exact immunogenetic analysis.

The male factor operates not only in transplanted skin and some tumors, but in thymus (Hirsch, 1957), lymph nodes (Feldman, 1958), lung, and salivary gland (Eichwald, Silmser, and Weissman, 1958). It is also present in male spleen, liver, and whole blood, since Eichwald, Silmser, and Weissman (1958) produced partial immunity against a male fibrosarcoma in females pretreated with these tissues.

In Table 6 we have summarized all available skin-graft data for 12 inbred strains. The male antigen appears to be widely distributed. While it behaves very uniformly in one strain (C57BL) and in one laboratory (Sachs and Heller, 1958), most sublines of C3H, DBA/2, Balb/c, and A/Jax react inconsistently.

The irregular response to male skin by females in these strains and in test hybrids has provoked many speculations: Relative weakness or complete absence of the antigen (Snell, 1956; Prehn and Main, 1956; Eichwald, Silmser, and Wheeler, 1957); endocrine differences between strains; intrastrain variation in the recipient's ability to respond toward the male factor; sex-limited autosomal inheritance (Short and Sobey, 1957); "an autosomally controlled replica of a Y-controlled antigen" (Michie and McLaren, 1958); and "hybrid vigor" determining the absence of male tolerance in "strong hybrids" or acceptance of male grafts by "weak hybrid" females (Eichwald, Silmser, and Weissman, 1958).

A tenable working hypothesis must do justice to the pertinent fact that some, but not all, females and males of the inconsistently behaving strains can transmit male compatibility to their daughters. *This transmission occurs not only within a so-called "weak" strain* but in outcrosses of weak to strongly reactive strains.

The seeming complexity of the data is resolved best by a cytogenetic extension of the hypothesis (Hauschka, 1955) that the male antigen is Y-linked: In C57BL/Ha mice, the antigen remains isolated on the Y chromosome. Rejection of male skin by females is therefore consistent. In other strains (e.g., C3Hf/Ha or Y/HeHa) female rejection of male tissue is inconsistent, female-to-female rejection has been observed, and complete compatibility may evolve

ling a general nucleic acid synthesis pattern." By-products of this activity may be sufficiently specific for "immunological detection" of the Y chromosome by rabbit antisera (Levit *et al.*, 1936). However, Fox and Yoon (1958) have shown a qualitative shift in antigenic pattern depending on the number of X chromosomes rather than the Y. XY flies produce a male antigen which disappears in XXY flies. In the discussion of their interesting data, they deplore the nonavailability of XXY mice for similar tests. However, the cytogenetic interpretation of our data would argue in favor of the existence of such mice in certain inconsistently behaving strains.

These suppositions now will have to undergo further immunogenetic and, above all, cytologic tests. The sex-linked histocompatibility system of mice merits considerable further exploration, for its genetic virtues are unique: It functions against an otherwise isogenic background, seems tied up with a chromosome which is morphologically distinguishable, and elicits detectable isoantibodies. Hence, it should be instructive as a model of antigenic differentiation in normal tissue, and antigenic instability in malignant cells.

Summary

The sex-linked histocompatibility system in mice has been analyzed further:

Male-to-female skin grafts within several inbred strains have given three types of response: 1. consistent rejection in C57BL/Ha and A129/Ha; 2. varying frequencies of compatibility in C3Hf/Ha and Y/HeHa; and 3 complete compatibility in DBA/2 and Y/HeHa subline 2

The male factor or "Y-linked antigen" was, however, present in all three types of mice, as proved by suitable outcrosses. Hence, female nonreactivity in classes 2 and 3 is attributed to the presence of the Y antigen in the male-compatible females, resulting from translocation or nondisjunction during spermatogenesis. Occasional rejections of female skin by females in group 2 fit this hypothesis.

Male tolerance was experimentally induced in females by x-irradiation, injection of male cells at birth, and (in only one case) by foster-nursing a female from a refractory strain on a male-tolerant nurse.

Isologous female anti-male sera contained weak but definite leukoagglutinins and leukocytotoxins; they did not agglutinate male red cells

Five tumors originating in C57BL/Ha male mice contained the male antigen, as shown by their transient growth inhibition in fe-

rapidly within a stock. Do such mice transmit compatibility to their daughters simply by transmitting the antigen which has become attached to the X chromosome or an autosome?

If antigenic Y material enters the rest of the genome, sex-linked histocompatibility would be at stake. Primary meiotic nondisjunction or translocation during spermatogenesis could initiate the sequence of events outlined in Figure 7.

Once the male antigen has become attached to the X chromosome or to an autosome, carriers of either sex would quickly disseminate it. Increasing numbers of females in such a "contaminated" population would accept male skin, for they could not react against an antigen present in their own somatic cells. Intra-uterine and milk influences are apt to induce phenotypic male tolerance, even among those daughters that have not acquired the male factor by genetic transmission. The progressive changes in graft behavior witnessed in our yellow strain (Table 3) are a case in point.

The nonchiasma type, firm end-to-end adherence established for the mouse X and Y chromosomes during meiosis (Sachs, 1955; Ohno, Kaplan, and Kinosita, 1957a, 1957b) would seem to favor occasional nondisjunction. This would give rise to XY sperm and thereby XXY females. Inbred strains of mice may differ in the tendency of their X and Y to be connected. Painter (1927) has found variations in the frequency of unconnected X and Y chromosomes at first meiotic metaphase in different types of mice. These two phenomena may well decide whether the male antigen remains firmly Y-linked and restricted to males—as it does in C57BL—or crosses the cytological sex barrier because of nondisjunction or asynapsis.

The allelic complexity known for other histocompatibility systems in the mouse seems not to be paralleled in the male factor. Combining our data with those of Eichwald, Silmser, and Weissman (1958), Bernstein, Silvers, and Silvers (1958) and Zaalberg (in press), we may conclude that C57BL, A/Jax, DBA/2, C3Hf/Ha, Y/HeHa, and CBA all have the same Y factor. The apparent absence of allelism and strain specificity is in keeping with the genetic inertness of the Y heterochromatin. It may even suggest that the male antigen is not the product of a true histocompatibility locus, but a property of the Y heterochromatin as a whole.

From the biochemical studies of Schultz and his colleagues (Schultz, 1956) on the Y chromosome in *Drosophila melanogaster*, it is apparent that genetic "inertness" means anything but synthetic inertness; for the Y heterochromatin is most "influential in control-

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males. Cytological comparison of male and female passage sublines in one of these neoplasms was suggestive of immunoselection, possibly against the Y chromosome or a translocated part thereof.

These results are discussed in relation to previously published data and hypotheses.

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The Chromosomal Status of Drug Resistant Sublines of Mouse Leukemia L1210

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Several different mechanisms have been proposed for the development of drug resistance in neoplasms. In thorough reviews, based in part on experience with mouse leukemia L1210, Law (1956, 1958) dismissed the possibilities of physiologic adaptation and directed mutation, and supported strongly the proposition that random mutation and selection by the drug of resistant mutants in the diverse cellular population constitute the mechanism by which stably resistant populations of neoplastic cells arise. Leukemic cells resistant to A-methopterin occurred in sensitive populations not previously exposed to antifolic acids (Law, 1952a). In experiments based on the bacterial "fluctuation test" (Luria and Delbrück, 1943), Law (1952b) observed great differences in response to A-methopterin after six passage generations among sublines originating from one subcutaneous implant of leukemia L1210, but consistent response to the agent in multiple tests of an individual subline. One of 10 clones of the hyperdiploid Ehrlich ascites tumor arising through single-cell transplantation (Quermer, 1955) showed reduced sensitivity to colchicine, indicating presence of resistant cells in the original population (Lettré, 1956).

Neither the concept of point mutation nor the possible biochemical

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lines The literature also includes reports, which need not be discussed here, on the presence or absence of evident chromosomal changes accompanying various physiological, nutritional, or biochemical differences in mammalian cell clones.

Procedure

Our investigations have been carried out on an ascitic parent line and nine sublines of mouse leukemia L1210. It may be mentioned that Law (1951) had observed no morphologic changes in L1210 cells transformed to A-methopterin resistance. The leukemia was known to be diploid or near-diploid (Law, 1956), but sensitive and resistant sublines of L1210 had not been compared cytologically.

Our parent line of L1210 was obtained from J. H. Burchenal in September, 1956, and since then it and its sublines have been carried in Bittner's D_2BC mice. Some of the experiments were made with DBA/2 mice.

The sublines have been developed by passage under continuous exposure to various chemotherapeutic agents. Subline IV was started early under treatment with A-methopterin at 3 mg/kg. We examined the chromosomes from transfer generations 31 and 42 after recovery from a frozen tumor bank.

Another group of sublines originated from an early branch off the parent line. At the 10th generation of this branch, subline X was started under exposure to 6-mercaptopurine at 40 mg/kg. We have examined chromosomes from the 57th and 59th passage generations, and from generation 71 after 10 generations of no treatment. Subline XI, started at the same time as subline X, was treated with a combination of amethopterin at 3 mg/kg and 6-mercaptopurine at 40 mg/kg. We examined chromosomes in generation 41.

The remaining sublines were taken off the main stem of the parent line (V). At the 10th generation, subline IX was begun under exposure to A-methopterin at 1.0 mg/kg, and subline VIII was started under 6-mercaptopurine at 20 mg/kg. We examined chromosomes of generation 45 of subline IX and of generations 47, 51, and 71 of subline VIII.

Two generations of the parent line later, subline III was begun under exposure to 40 mg/kg 6-mercaptopurine. Chromosomes were examined from generations 53, 59, and 80, as well as from generation 79 in a subbranch untreated for nine generations.

Subline XII was begun at the 15th generation of the parent line.

or other means by which a genetic change produces drug resistance is within the province of this paper. We have proposed to investigate the possibility of genetic change in the sense of gross chromosomal realignment by the comparatively crude approach of karyotype analysis. The present paper is, however, little more than an extension of the recent examination of this subject by Hauschka (1958).

The study of Hauschka (1958) was on seven resistant or drug-dependent sublines of various mouse neoplasms and their five sensitive strains of origin. Four chemotherapeutic agents were involved. In four of the seven resistant sublines, no chromosomal difference distinguished the derived line from the sensitive parent line. However, one of these four may have concerned a nongenetic effect, because resistance to A-methopterin in a treated subline of plasma cell tumor 70429 reverted to sensitivity in the absence of the agent (Law, 1958). In three instances, Hauschka found that changes in chromosomal number and morphology occurred in the resistant tumor. The development of resistance to DON in two sublines of a mast cell tumor was accompanied by no evident chromosomal change in one subline but a more pronounced mode of chromosome number and two new marker chromosomes in the other.

Hauschka (1958) pointed out that his data on chromosomes, in which he analyzed a number of transfer generations after the development of resistance, did not completely justify the inference that the observed chromosomal changes caused the development of resistance. However, the gross chromosomal changes could contribute to variability in response of the tumor cells beyond that attributable to point mutation.

An earlier study (Hirono and Yokoyama, 1955) demonstrated no chromosomal differences between the original sensitive Yoshida sarcoma and a derivative subline resistant to nitrogen mustard N-oxide.

The chromosome number mode of the "diploid" Ehrlich ascites carcinoma may have shifted in the development of a colchicine-resistant strain (Lettré, 1958). Counts on the original sensitive strain (Bayreuther, 1952) lay 93 per cent in the chromosome number range 41 to 50, but counts made some years later by Querner on a strain resistant to N-methylcolchicamide gave 48 per cent in the range 31 to 40 and only 39 per cent in the range 41 to 50.

In summary, then, the available literature gives a number of instances in which no chromosomal changes distinguished drug-resistant or drug-dependent tumor sublines, and about as many instances in which there were karyotypic differences from the parent

TABLE 1. Chromosomes of L1210 V (Parent Line)
Generations 62, 81, 83

Number	Frequency	Markers* in Near-Diploids Set	Frequency
36 or less	4	S	25
		S, m	59
37	3	S, mm	20
38	6	S, mmm	4
39	13	S, M, m	4
40	30	S, M	1
41	32	m	2
42	15	mm	2
43	10	none	6
44	4	Total	123
45 or more	6	Modal Cell	
80, 87	2	(38–40)T, 1S, 1m	
Total	125		

* *Chromosome types are designated thus T, telocentric, M, metacentric; S, submetacentric, m, minute*

cells contained a chromosome of medium length with a medially located centromere The most common combination of marker chromosomes was one example of the long submetacentric plus one minute chromosome The "modal cell," in consequence, displayed from 38 to 40 telocentric chromosomes plus one submetacentric plus one minute chromosome This parent line V exhibited notably more variability in chromosome number and in marker chromosomes than did some of the drug-resistant sublines.

The A-methopterin-resistant sublines IV, IX, and XI had some points of cytological agreement (Table 2) Both generations of subline IV examined showed a modal class of figures with 42 chromosomes, which typically included 41 telocentric chromosomes and one minute Although 94 per cent of subline IV cells contained minute chromosomes, only a minority of mitotic figures in sublines IX and XI did so The modal figures in these two sublines contained 40 telocentric chromosomes

The sublines developed by exposure to 6-mercaptopurine included III, VIII, and X. Sublines III and VIII resembled the parent line V, while subline X resembled the A-methopterin-exposed sublines

Subline XII, treated with azaserine at 20 mg/kg, was examined in generation 33.

From the 53rd generation of the parent line came the last two sublines. Subline XIIIs, under exposure to 25 mg/kg of 5-fluorouracil in saline, was examined in generations 5 and 23. Subline XIIIr, for which the 5-fluorouracil vehicle was peanut oil, was examined in generations 6 (unsuccessfully) and 27. In the last case, the examination was made in an untreated mouse, but the cells had been treated for the previous 26 passage generations.

Because average survival times of mice carrying this ascitic L1210 leukemia and its various sublines ranged from 9 to 13 days, mice for chromosomal studies normally were taken on the fifth day after inoculation and injected intraperitoneally with 20 micrograms of colchicine (Hauschka and Levan, 1958) in 0.2 cc. Gey's balanced salt solution. Some 14 to 16 hours later each mouse received an intraperitoneal injection of 1 cc. Eagle's basal medium, to aid in our recovery of the cells, and was killed by cervical dislocation. The ascitic fluid was removed and added to several times its volume of a warmed hypotonic Gey's solution (Hsu and Pomerat, 1953). After some 10 minutes' exposure and a light centrifugation, the fluid was decanted from the cells and orcein stain in 60 per cent acetic acid was added. The cells were pressed out under cover glasses and sealed in with Gurr's compound

The cells were examined with phase-contrast oil immersion microscopy, and chromosome counts were made with the aid of freehand drawings and occasional photographs.

Results

Examination of three specimens of the parent line L1210 V revealed a broad modal group peaking at 41 chromosomes per figure, with almost as many cells having 40 chromosomes (Table 1). Some 80 per cent of the figures had from 39 to 43 chromosomes. There was a small representation of cells with approximately 80 chromosomes. Most of the cells contained marker chromosomes, the most prominent being a long element with submedian centromere and a long arm about three or four times the length of the short arm. This submetacentric chromosome also occurred commonly in sublines III, VIII, XIIIs, and XIIIr Of 123 near-diploid cells studied in line V, 113 contained the long submetacentric. From one to three minute chromosomes occurred in about 75 per cent of the cells, and a few

TABLE 3. Chromosomes of 6-Mercaptopurine-treated Sublines III and VIII

Number	Frequency III**	III_{19}	VIII
36 or less	3	1	1
37	1	4	1
38	1	3	3
39	9	10	12
40	57	16	27
41	11	12	14
42	6	2	9
43	0	1	8
44	0	0	2
45 or more	1	1	3
78, 79	2	0	1
115	0	1	0
Totals	91	51	81

Markers* in Near-Diploids Set	Frequency III**	III_{19}	VIII
S, m	71	34	49
S, mm	6	7	11
S, mmm	0	2	1
SS, mmm	0	1	0
S	11	5	12
SS	0	0	1
S, M	0	0	2
m	0	1	4
none	1	0	0
Totals	89	50	80
Modal Cell			
III 38T, 1S, 1m			
VIII 38T, 1S, 1m			

* Chromosome types are designated thus: *m*, minute, *M*, metacentric; *S*, submetacentric; *T*, telocentric.

** This column contains a summation of data from the continuously 6-mercaptopurine-exposed generations 53, 59, and 80 of subline *III*, while III_{19} was a ninth-generation untreated specimen

plus one minute chromosome. Unfortunately, a treated specimen of generation 71 was not examined.

Subline XII was examined in its 33rd passage generation under continuous treatment with azaserine (Table 5). The modal cell contained 40 telocentric chromosomes and one minute. The long submetacentric was seen in three cells of the 41 examined.

Of the recently developed sublines resistant to 5-fluorouracil, $XIII_s$ had a mode of 39 to 41 chromosomes, one of these being the long submetacentric. The spread of the mode as given in Table 5 resulted from a downward shift from a mode of 41 chromosomes in generation 5 to a mode of 39–40 in generation 23. Subline $XIII_P$ was examined in generation 27, in a mouse that was not treated with 5-fluorouracil in this last generation of the leukemia subline, because of difficulties with the peanut oil vehicle in the preparation of slides. The modal chromosome number was 40, the long submetacentric was present in every cell examined, and a minute chromosome was noted in 70 per cent of the cells.

TABLE 2. Chromosomes of A-methopterin-treated Sublines

Number	Frequency IV	IX	XI**
35	0	1	1
36	0	2	0
37	1	1	2
38	0	4	4
39	0	2	2
40	4	6	14
41	16	5	4
42	38	2	5
43	15	1	4
44 or more	3	1	3
82–85	5	0	0
98	0	0	1
Totals	82	25	40

Markers* in Near-Diploids

Set	Frequency IV	IX	XI**
m	65	8	12
mm	6	1	1
S, m	1	0	0
none	5	16	26
Totals	77	25	39

Modal Cell
IV: 41T, 1m
IX: 40T
XI: 40T

* Chromosome types are designated thus: *m*, minute; *S*, submetacentric, *T*, telocentric

** Subline XI was exposed to 6-mercaptopurine as well as to A-methopterin

from a chromosomal standpoint. Subline III contained a sharp peak of figures with 40 chromosomes, of which 38 were typically telocentric, one was the long submetacentric, and one was a minute chromosome (Table 3). Nearly 99 per cent of the figures examined in subline III contained the long submetacentric chromosome. This was also true for the 79th generation of subline III, examined in a subsidiary line in which exposure to 6-mercaptopurine had ceased with the 70th passage generation. In this last case, the mode at 40 chromosomes was not so pronounced.

Subline VIII also had a mode of 40 chromosomes, and the most common set of marker chromosomes included the long submetacentric and one minute. There were other sets of markers, and two figures of 81 examined appeared to contain one metacentric chromosome each, in addition to a submetacentric.

Subline X was different (Table 4). Generations 57 and 59 had a modal class of mitotic cells containing 40 telocentric chromosomes. *Only about a third of the mitoses had from one to three minute chromosomes.* In a 10th-generation untreated control of generation 71, the commonest mitotic figure had 40 or 41 telocentric chromosomes

TABLE 5 Chromosomes of Sublines Developed under Azaserine (XII) or 5-Fluorouracil ($XIII_S$ and $XIII_P$)

Number	Frequency XII	Frequency $XIII_S$	Frequency $XIII_P$
36 or less	1	5	3
37	0	1	1
38	1	3	6
39	4	7	12
40	7	8	16
41	10	8	8
42	7	5	3
43	6	3	1
44	4	0	0
47	0	1	0
48	1	0	0
83, 87	0	1	1
Totals	41	42	51

Markers* in Near-Diploids

Set	Frequency XII	Frequency $XIII_S$	Frequency $XIII_P$
S	0	26	15
S, m	2	11	35
S, mm	1	1	0
m	19	0	0
mm	8	0	0
mmm	1	0	0
none	10	3	0
Totals	41	41	50

Modal Cell
XII 40T, 1m
$XIII_S$: (39–40)T, 1S
$XIII_P$ 38T, 1S, 1m

* Chromosome types are designated thus *m*, minute, *S*, submetacentric, *T*, telocentric.

treated control from line III had six marker classes as opposed to *four* classes in the treated specimens of the subline. The 10th generation untreated control of subline X had four such classes, as did the treated specimens, but the markers occurred in 70 *per cent* of the cells instead of 36 per cent. The treated specimens of sublines III and X had sharper chromosome number modes than the untreated had Of course, the sublines may have had more variability in chromosome numbers and markers than was expressed in mitotic cells, as the results of Freed and Hungerford (1957) indicate for sublines of the Ehrlich carcinoma. However, the fact of their mitotic rarity would tend to diminish even more their proportional representation in the population in later cell generations.

It should be emphasized that the small number of specimens, small number of chromosome sets analyzed, and spotty selection of generations used in this preliminary survey render the relations just stated merely suggestive. The points at issue should be studied in specific experiments.

From a chromosomal standpoint, the sublines studied may be divided into two groups The long submetacentric chromosome of

TABLE 4. Chromosomes of 6-Mercaptopurine-treated Subline X

Number	Frequency $X_{57, 59}$**	X_{71}†
36 or less	0	5
37	1	1
38	0	2
39	6	3
40	36	1
41	16	15
42	9	12
43	1	7
44	0	3
45 or more	1	4
Totals	70	53

Markers* in Near-Diploids

Set	Frequency $X_{57, 59}$**	X_{71}†
m	15	25
mm	9	9
mmm	1	3
none	45	16
Totals	70	53

Modal Cell
$X_{57, 59}$: 40T
X_{71}. (40–41)T, 1m

* Chromosome types are designated thus *m*, minute, *T*, telocentric
** Pooled data of transfer generations 57 and 59 under continuous treatment with 6-mercaptopurine
† Data from a 10th-generation untreated leukemic ascites of transfer generation 71

Discussion

The findings suggest a greater chromosomal variability, with perhaps less pronounced chromosome number mode, in the parent line V than in the drug-resistant sublines individually, although the greater number of cells examined in line V may be partly accountable. A greater variability of the parent line would suggest that continuous treatment with the several antimetabolites had selected resistant genotypes, insofar as their existence may be inferred from the different karyotypes (Ford, Hamerton, and Mole, 1958), in the chromosomally varied and varying parent population. To the extent analyzed in this study, the stem cell karyotypes of the sublines all appeared to be present in the parent line V. The rapidity with which mutant genotypes in leukemic cell populations can replace the parent genotype under the selective pressure of antimetabolites may be gathered from the calculations of Skipper, Schabel, Bell, Thomson, and Johnson (1957).

There was variability in markers as well as in numbers of chromosomes. Line V displayed nine different combinations of marker chromosomes or their absence, more than any subline, although subline VIII approached it with seven classes. The ninth generation un-

apparent absences of such correlation. The relation between A-methopterin resistance and lack of the large submetacentric marker chromosome has a low probability of being fortuitous. Thus, of the parent line and nine sublines, the five in which the submetacentric chromosome is common are sensitive to A-methopterin, while the five in which this marker is absent or rare are resistant or only slightly sensitive to A-methopterin. If these data are treated in 2 × 2 contingency tables according to Finney (1948), the proportion of resistants without submetacentrics (5/5) is found to exceed the proportion of sensitives without submetacentrics (0/5) significantly at a probability level of 0 01. The same conclusion is reached for the proportion of sensitives with the submetacentric (5/5) with respect to the proportion of resistants with the marker (0/5).

However, resistance to 6-mercaptopurine, to azaserine, to 5-fluorouracil, to 8-azaguanine, or to mitomycin is not accompanied by distinctive cytological peculiarities in the sublines, so far as the present study goes. A more detailed study might reveal some significant correlations. For instance, there were probably some chromosomes longer than the longest of the normal murine complement, as well as some chromosomes shorter than the shortest of the normal complement (and still not listed as minutes) in these L1210 plates, such as Ford, Hamerton, and Mole (1958) found in their near-diploid reticular neoplasms of the mouse. Such chromosomes might well differ from subline to subline. Evidence regarding them and other indications of translocations and cryptostructural rearrangements and losses (Levan, 1956; Hauschka, 1958) might be produced by precise idiogram analysis.

Summary

Chromosomes were studied in nine variously drug-resistant sublines of the near-diploid L1210 leukemia of the mouse. The five sublines resistant to A-methopterin lacked a large submetacentric chromosome that characterized the modal complements of the parent line and the other four sublines, all of which were sensitive to A-methopterin. No consistent cytological changes accompanying resistance to 6-mercaptopurine, azaserine, 5-fluorouracil, or mitomycin were noted

ACKNOWLEDGMENTS

The work reported in this paper was supported in part by grants from the American Cancer Society, New York, N.Y , and by Grant CY-3192

the parent line V also occurred in the modal figures of sublines III, VIII, XIIIs, and XIIIr. This chromosome was not seen in sublines IX, X, and XI; it was seen, doubtfully, once in 98 figures of subline IV, and was seen in only three figures of 41 analyzed in subline XII.

Roughly, the subline patterns of drug sensitivity and drug resistance fall into somewhat similar groupings.

The members of the first group, which lack the long submetacentric chromosome, show some points of strong resemblance in drug resistance. Thus sublines IV, IX, and XII are essentially resistant to A-methopterin at 3 mg/kg; while sublines X and XI show only slight sensitivity (Hutchison, 1958). Although sublines IV and IX were selected for A-methopterin resistance and XI for resistance to A-methopterin plus 6-mercaptopurine, X was selected for resistance to 6-mercaptopurine and XII for resistance to azaserine. All these sublines are resistant to 6-mercaptopurine at 40 mg/kg, as are III and VIII, and essentially so to 8-azaguanine at 90 mg/kg. Sublines IV, IX, and XII, but not X or XI, resist azaserine at 20 mg/kg (D. J. Hutchison, unpublished). They are variously sensitive to 5-fluorouracil at 25 mg/kg, as are all of the other lines except XIIIs and XIIIr. Their sensitivity to mitomycin at 2 mg/kg (D. J. Hutchison, unpublished) approximates that of the parent line V, except for subline IX, which is resistant. The one consistent relation, then, is that between resistance to A-methopterin and absence of the large submetacentric.

Sublines III, VIII, XIIIs, and XIIIr, as well as line V, are sensitive to A-methopterin and possess the large submetacentric chromosome. There the resemblance ceases between sublines III and VIII, on the one hand, and the sublines XIII, on the other. The sublines XIII are sensitive to 6-mercaptopurine, but III and VIII are not, while the latter are sensitive to azaserine and to 5-fluorouracil, unlike the sublines XIII. Sublines III and VIII are the two lines most inhibited by mitomycin (D. J. Hutchison, unpublished).

Sublines III and VIII resemble one another more closely than they resemble the parent line or any other subline in their responses to the chemotherapeutic agents tested They are also similar chromosomally. However, the chromosomal constitution of their modal cells apparently is included within the broader modal constitution of the parent line V and is approximated by that of XIIIr.

In short, study of these sublines of mouse leukemia L1210 has revealed some points of good correlation between chromosomal constitution and response to a chemotherapeutic agent, as well as some

apparent absences of such correlation. The relation between A-methopterin resistance and lack of the large submetacentric marker chromosome has a low probability of being fortuitous Thus, of the parent line and nine sublines, the five in which the submetacentric chromosome is common are sensitive to A-methopterin, while the five in which this marker is absent or rare are resistant or only slightly sensitive to A-methopterin. If these data are treated in 2 × 2 contingency tables according to Finney (1948), the proportion of resistants without submetacentrics (5/5) is found to exceed the proportion of sensitives without submetacentrics (0/5) significantly at a probability level of 0.01. The same conclusion is reached for the proportion of sensitives with the submetacentric (5/5) with respect to the proportion of resistants with the marker (0/5).

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Acknowledgments

The work reported in this paper was supported in part by grants from the American Cancer Society, New York, N Y., and by Grant CY-3192

from the National Cancer Institute, Public Health Service, Bethesda, Md., to the Sloan-Kettering Institute for Cancer Research.
Dr. Biedler was Sloan Predoctoral Fellow, 1956–1958.

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BERTNER FOUNDATION

The Roles of Virus and Host in Determining the Host Reaction to the Fibroma-Myxoma Virus Complex

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In some virus diseases analysis of the host-virus relationship is simple enough to be amenable to quantitative consideration. Several of these situations have served as topics for discussion in recent years However, in many cases, due to the complexity of numerous interacting factors and to variations in both the host and the virus, the resulting disease picture has been too complicated to deal with on a strictly quantitative basis as regards host-virus relationship. This is true of the fibroma-myxoma disease complex, and because of this, I shall have to resort to the use of qualitative differences in indicating the manner in which variations in either the virus or the host can influence the disease expression shown by the host.

Infectious myxomatosis is an acute, highly fatal disease of domestic rabbits, caused by the myxoma virus. Following subcutaneous inoculation of a susceptible rabbit with myxoma virus, a soft tumorlike mass develops at the inoculation site within three or four days. This increases rapidly in size, and on cut section is pink and gelatinous and has the gross appearance of a myxoma. At about the fifth day the eyelids swell because of inflammation and edema, and there is edema of the mucous membranes and skin at the anal and genital orifices. The animal during this period is febrile The disease progresses rapidly, the eyelids swelling completely shut, and the nasal orifices swelling so that breathing is made difficult. Multiple myxomatous tumors may also appear over other parts of the body The disease is uni-

to myxoma virus was achieved without the formation of demonstrable myxoma virus-neutralizing antibodies in the sera of the fibroma-recovered rabbits, although after challenge with myxoma virus they did develop such antibodies.

The immunological relationship between the two viruses in the reverse direction was more difficult to determine because rabbits so seldom recovered from myxoma virus infection. However, Sir Charles Martin (1936) in a large series of myxoma cases had several survivors and tested the active and passive immunity of five of these to fibroma virus. He found that the sera of all five of these myxoma-recovered rabbits neutralized not only myxoma virus but fibroma virus as well, and that all five rabbits were solidly resistant to infection with fibroma virus.

The immunological findings just outlined suggested that although the fibroma and myxoma viruses were not identical, there was at least partial duplication of the antigenic components comprising the two agents Furthermore, the suggestion was strong that the myxoma virus contained all of the antigenic components present in the fibroma virus, or at least all of those essential to the production of a complete fibroma virus immunity. On this basis then, the incomplete protection afforded by fibroma virus against myxoma virus infection seemed to be an indication that fibroma virus might be antigenically only a partial replica of myxoma virus In short, it appeared possible that the myxoma virus was comprised of fibroma virus plus certain additional components and that fibroma virus might, *therefore*, bear an analogous relationship to myxoma virus as that borne by "rough" to "smooth" forms of certain bacterial species.

Two approaches to determine the correctness of the possibility were apparent. One of these, and the one that I thought should be exploited first, was to attempt, by various manipulations, to convert myxoma virus to fibroma virus. I tried to do this by passing myxoma virus serially through wild cottontail rabbits (Shope, 1936a) and through fibroma-recovered domestic rabbits (Shope, 1936b). Neither of these procedures had the slightest effect in changing myxoma virus to fibroma virus.

The other approach, analogous to Griffith's (1927–28) transformation experiments with pneumococci, was to attempt to convert fibroma virus to myxoma virus. This was carried out successfully by Berry and Dedrick (1936). These investigators, by injecting mixtures of proper proportions of heat-inactivated myxoma virus and active fibroma virus into domestic rabbits, transformed fibroma virus to

formly fatal, death occurring usually on from the seventh to the twelfth day following inoculation. Myxomatosis was first recognized by Sanarelli (1898) among rabbits in his institute at Montevideo in 1896, and he demonstrated that it was caused by a filtrable virus—one of the very early ones to be recognized.

In contrast to the regularity with which myxoma virus kills domestic rabbits, the fibroma virus causes an entirely benign ailment in adult domestic rabbits (Shope, 1932a). Administered intracutaneously, intratesticularly, or subcutaneously, the fibroma virus gives rise to a rapidly growing tumorlike mass that is firm and white on cut section and that histologically resembles a fibroma in many respects. The tumor increases progressively in size for about a week, remains stationary for about the same time and then slowly regresses, finally to disappear completely. It never kills adult domestic rabbits and never causes manifest signs of generalized illness. In the wild cottontail rabbit, which is the natural host of the fibroma virus, the tumors almost always occur on the feet and, in contrast to their evanescence in domestic rabbits, may persist for as long as ten months before eventually regressing.

Thus, in the cases of the fibroma and myxoma viruses, we have two agents which, when considered superficially, appear to cause entirely different diseases in adult domestic rabbits, the one, that induced by the myxoma virus, is severe and always fatal; the other, that induced by the fibroma virus, is benign and never fatal. The only feature that the two diseases have in common is that in both a tumorous mass, comprised of proliferating connective tissue cells, results at the local site of virus injection. It was this similarity that first called attention to the possibility that the two viruses might be related (Shope, 1932b).

On further exploration of the possibility that a relationship might exist, rabbits that had recovered from infection with fibroma virus were found to be relatively resistant to myxoma virus. This cross-resistance was not of the absolute type that would have existed had the two viruses been antigenically identical. Rather, it was of a partial type that might be induced by a closely related but not identical agent. Thus, when fibroma-recovered rabbits were inoculated with myxoma virus, they developed a localized myxomatous tumor at the site of injection, just as do fully susceptible rabbits. However, instead of progressing to a fatal termination, as myxomatosis always does in susceptible rabbits, the fibroma-recovered rabbits ordinarily underwent a relatively benign illness and almost never died. This resistance

the picture of the disease caused by this virus in the two types of hosts were, briefly, as follows In domestic rabbits the incubation period, that is, the time between inoculation and beginning tumor formation, was from two to five days; whereas in the cottontail the incubation period varied between 10 and 15 days. Once initiated, the tumors grew and reached their maximum size much more rapidly in domestic than in cottontail rabbits Ordinarily by the tenth day after inoculation, the fibromas in domestic rabbits had attained their maximum size; whereas in cottontails this was not reached until between 40 and 50 days after inoculation. After reaching their maximum size, the subsequent course of the tumors in the two species was different In domestic rabbits the fibromas occasionally persisted without noticeable gross change for as long as two weeks, but more usually they persisted for only six or seven days and then underwent rapid regression Regression was usually complete by three weeks after infection, and even in the more persistent cases it was complete by five weeks. In the cottontail, however, the fibromas, after once reaching their maximum size, usually persisted unchanged for long periods of time, and ordinarily presented a normal healthy "fleshy" appearance 12 to 14 weeks after infection. I have not, in my series, purposely held rabbits for extended periods of time to see how long the tumors would persist, but Kilham and Dalmat (1955) have recorded that one of their experimentally induced fibromas in a cottontail was still intact and contained virus 10 months after infection. The fibromas in cottontails eventually disappear, but I cannot, from my experiments, state the time ordinarily required; nor is it clear just how much influence in initiating regression is exerted by trauma resulting from injuries during cage confinement. At any rate, the course of fibromatosis is different in the two species of rabbits, being more rapid in inception, progression, and regression in the domestic rabbit than it is in the wild cottontail. In addition, though histologically the growths in both species appear to be composed of proliferating connective tissue cells, those in the cottontail show a thickening of the overlying skin epithelium with cytoplasmic inclusions in individual epithelial cells, a condition not usually seen in the domestic rabbit fibromas It is apparent that the fibroma virus reacts quite differently with its experimental host, the domestic rabbit, than it does with its natural host, the wild cottontail rabbit

But the host-virus relationship, even within the domestic rabbit, may be varied greatly if one resorts to the use of very young rabbits as Duran-Reynals (1940) has done. This investigator found that

myxoma virus. The transformed myxoma virus bred true on further serial passage and behaved in all respects as typical myxoma virus. The Berry-Dedrick transformation has been confirmed repeatedly by other investigators, and recently Kilham (1957) has succeeded in effecting it in tissue cultures of rabbit cells.

The Berry-Dedrick phenomenon thus established as a fact the suspected relationship between the fibroma and myxoma viruses and indicated the unity of the two agents. No one, of course, knows which agent represents the original type virus, i.e., whether the South American myxoma virus should be considered as a virulent offshoot of the North American fibroma virus or whether the latter should be thought of as a benign derivative of the South American agent. However, the important point to establish for the purpose of this discussion is that the fibroma and the myxoma viruses are but the benign and virulent varieties, respectively, of the same virus.

There are two other varieties of the virus that will be alluded to in discussing host-virus relationships between this agent and rabbits. These are the IA (*inflammatory*) *strain of fibroma virus* (Andrewes, 1936) and neuromyxoma virus (Hurst, 1937a). The IA fibroma virus apparently arose "spontaneously" as a mutant of the original (OA) strain of fibroma virus and was segregated by a series of circumstances favorable to its isolation. The neuromyxoma virus, however, was derived by serial cerebral passage of myxoma virus in domestic rabbits. Both of these variant strains seemingly bred true once they were established.

Various disease expressions can be achieved by merely varying the interacting virus variety and the host in the host-virus relationship. I shall deal first with the various manifestations that can be brought about by the classical fibroma virus as it comes directly from the fibromas of wild cottontail rabbits. The hosts to be employed, in varying the host-virus relationship, will be adult and baby domestic rabbits, cottontail rabbits, and tarred domestic rabbits

Host-Virus Relationships of Fibroma Virus

The original strain of fibroma virus (OA strain)--the one with which most of the published work has been conducted--was obtained initially from tumors on the feet of a wild cottontail rabbit shot in New Jersey (Shope, 1932a). This virus produced tumors in other cottontail rabbits that were like the tumors from which it had been obtained originally. In adult domestic rabbits, it induced fibromatous tumors when inoculated into or beneath the skin. The differences in

just as did normal rabbits. However, the course of the fibromas in the tar-injected animals was quite different from that in the controls, and instead of regressing after 15 to 20 days, as did the growths in the control animals, those in the tarred rabbits lasted for months in some cases. Furthermore, in the tumors in the tarred rabbits, the fibroma cells were more closely packed and lacked the inflammatory cells usually seen in normal fibromata. Because of its greater cellularity and the absence of inflammatory cells inside it, the cutaneous fibroma in tarred rabbits appeared more like a neoplasm than did the ordinary fibromas. In addition, in almost all cases of persisting cutaneous tumors in tarred rabbits, changes in the overlying epidermis were present. The epithelium showed thickening due to an increased number of enlarged cells, and epithelial projections extended downwards into the fibromatous tissue. Many of the cells in the epidermis, and especially in the downward projections, had an accumulation of small acidophilic granules in their cytoplasm, staining red with eosin. Recalling the earlier description of the course and the histological appearance of experimentally induced fibromas in cottontail rabbits, one will note the striking resemblance of fibromas in tarred domestic rabbits to those in cottontails. The reaction to fibroma virus in rabbits *treated with tar shows not only the same clinical course, but also the* same histological reaction as that seen in the wild cottontail rabbit. It would thus appear that tar in some manner converts the adult domestic rabbit to a host that, so far as the host-virus reaction is concerned, behaves strikingly like the wild cottontail rabbit to fibroma virus administered intracutaneously.

Mention has been made, in connection with the account of Duran-Reynals' experiments with suckling rabbits, that fibroma virus given intravascularly to adult domestic rabbits was without visible effect. Nothing was to be seen, except perhaps a small fibroma at the site where the needle entered the vein. However, in tarred rabbits the picture is quite different. In such animals, fibroma virus given intravenously results almost invariably in the formation of a fibroma in the thigh muscle where the tar had been deposited a few days earlier. Furthermore, in tarred rabbits, a generalized fibromatosis commonly develops and may be fatal. The generalized fibromas are scattered over the skin and diffusely in the subcutaneous tissue. They show an affinity for periosteal tissue and may be attached to the ribs or to the vertebral column or even to the bones of the legs. In several of the animals in the series of Ahlström and Andrewes, fibroma nodules were seen in the mouth as ulcerated tumors at the base

suckling rabbits reacted quite differently from adult animals to fibroma virus. For instance, although the fibroma virus was entirely without observable effect when given intravascularly to adult rabbits, Duran-Reynals found that it caused a rapidly fatal illness when similarly administered to suckling rabbits. The disease in the baby rabbits was primarily an inflammatory one, causing multiple hemorrhages in the viscera and bloody transudations in the pericardial, pleural, and peritoneal cavities. Histologically, there were degenerative cellular changes in the liver and kidneys. In addition, nests of proliferating fibroblast-like cells were found, apparently arising from the adventitia of blood vessels in various of the tissues and organs of the body. Whorls of fibroma-like cells frequently were observed occluding the vessels from which they originated.

When the virus was given subcutaneously to baby rabbits, it also produced a disease expression quite different from that in adult domestic rabbits. The local lesion was much more extensive and tended to be more inflammatory than proliferative, although it did contain isolated fibroblast-like cells in small numbers. Satellite growths similar to the primary lesion appeared in the surrounding skin. There were degenerative visceral lesions. All of the rabbits developed what appeared to be metastatic fibroma lesions in regional lymph nodes, in the abdominal viscera, and in the diaphragms and intercostal muscles. The disease was regularly fatal in from two to four weeks and had many of the earmarks of a highly malignant neoplastic process. Duran-Reynals called attention to certain similarities between the behavior of fibroma virus in suckling rabbits and myxoma virus in adult domestic rabbits. However, the virus that had caused the regularly fatal metastatic disease in the sucklings still retained the properties, for adult animals, of fibroma virus, and on back passage to adults produced only benign fibromatosis. It is readily evident from the experiments of Duran-Reynals that age of the host can serve as a major factor in determining the character of the virus-host reaction in fibromatosis.

Factors other than those associated with age also can play a role in determining the disease expression of the domestic rabbit host to fibroma virus. Thus, Ahlstrom and Andrewes (1938) made the interesting discovery that a single intramuscular or subcutaneous injection of a small amount of horizontal retort tar altered the reactivity of adult domestic rabbits to fibroma virus. Rabbits that had received the tar injection five to ten days before inoculation intracutaneously with fibroma virus developed fibromas at the site of virus injection

stage progresses to local fibroma formation dominated by the accumulation of large numbers of young fibroblast-like cells showing numerous mitotic figures In the case of the IA virus infections, however, the early reaction stage is followed by one in which there is a massive accumulation of lymphocytes in the interstitial tissues Thus, instead of causing a tumorlike swelling and fibroma formation, injection with the IA virus induces only inflammation with swelling, followed by atrophy in an inoculated testicle; or some local thickening of the skin, followed by necrosis, as a result of subcutaneous or intracutaneous injection. The disease expressions induced by these two varieties of fibroma virus in the same host are thus strikingly different.

Another finding that illustrates the differing relationship to a common host exhibited by the IA and OA fibroma viruses is that of Ahlström and Andrewes (1938) in tarred rabbits. Here, as noted earlier, the intravenous injection of OA virus into rabbits that had had a previous intramuscular injection of tar resulted in the appearance of a persistent generalized fibromatosis that was *sometimes* fatal Tarred rabbits injected intravenously with IA virus, however, failed to react and remained completely normal. Therefore, it seems that two immunologically identical varieties of the fibroma virus are capable of exhibiting quite different host-virus interactions, and that both the host and the virus may play important roles in determining the disease expressions resulting from their interaction.

Host-Virus Relationships of Myxoma Virus

Now the more virulent member of the fibroma-myxoma virus complex, the myxoma virus, will be considered, and some of its relationships to its hosts under varying conditions will be explored. This virus, which, as indicated earlier, is a regular killer of domestic rabbits, can be altered to one with less regularly fatal pathogenicity. Hurst (1937a), starting with fully lethal myxoma virus, was able, by serial cerebral passage in rabbits, to obtain a virus with pathogenic properties somewhat intermediate between those of classical fibroma and myxoma virus This variant, which Hurst termed neuromyxoma virus, caused a disease which was less severe than myxomatosis and which seldom ended fatally The lesions were generalized as in myxomatosis, but individually resembled more the lesions of fibroma. The result produced by neuromyxoma virus was strongly reminiscent of that obtained by Ahlström and Andrewes in tarred rabbits with fibroma virus.

of the upper incisors. In other cases there were fibromatous masses about the nose, eyes, and anus, strongly reminiscent of the localization of the lesions seen in rabbits with myxomatosis. The course of intravenously induced fibromatosis in tarred rabbits was ordinarily quite prolongued. Moreover, many of the tumors, when examined histologically, had the appearance of real neoplasms: They appeared to be composed of one type of cell, there were many mitoses, and the inflammatory reaction which accompanies the lesions in normal rabbits was absent. Fibroma virus, typical in all respects, could be recovered from tarred rabbits with persistent fibromas many months after their initial infection. It is quite apparent that Ahlström and Andrewes very markedly altered the domestic rabbits' reactivity to intravenously administered fibroma virus by a single small injection of tar. *They thus converted a host-virus relationship from one in* which the virus had no visible effect upon its host to one in which a violent disease developed, sometimes even leading to the death of the host. In one instance (Andrewes and Ahlström, 1938), a transplantable sarcoma arose in a rabbit inoculated with tar and fibroma virus, though virus was never detectable in this malignant tumor by any tests that were applied.

So far only the influence of the host in the host-virus relationship has been discussed. The point has been made that, using the OA strain of the fibroma virus, four differing disease expressions can be elicited by this single variety of virus in four varying host situations—adult domestic rabbits, adult wild cottontail rabbits, suckling domestic rabbits, and tarred domestic rabbits. Now we may consider the variations in disease expression that can be attained when one employs another variety of fibroma virus in domestic rabbits, the so-called IA (inflammatory) strain (Andrewes, 1936).

The IA strain of fibroma virus is a variant that arose seemingly spontaneously *from the OA strain Immunologically the two* strains are identical and readily cross-protect one against the other. However, the disease expressions they induce in adult domestic rabbits are different. As previously described, the OA strain of virus induces a marked fibroblastic proliferation at the site of injection when it is administered intratesticularly, subcutaneously, or intracutaneously. The IA virus, however, produces only inflammatory reactions where it is injected. The early stages of disease expression are the same for both viruses—hyperemia, local edema of the interstitial tissues, and some exudation of polymorphonuclear cells at the sites of *injection.* In the case of infections induced by the OA virus, this early reaction

to a grossly normal state or to one of atrophy, depending upon the severity of the preceding reaction.

None of the cottontails in the series that I have injected with myxoma virus by any route have died, and none have appeared ill. The host reaction to the virus is thus markedly different in the cottontail from that in the domestic rabbit, where death following infection is the rule. Despite the fact that the cottontail is affected but lightly by myxoma virus, passage of the virus in cottontails does not alter the pathogenicity of the virus for domestic rabbits. Myxoma virus that was transferred through a series of 10 consecutive passages in cottontails, and in cottontail tissues for 140 continuous days during these passages (Shope, 1936a), was unchanged, and killed domestic rabbits just as typically as had the myxoma virus with which the series was initiated

Intermediate between the benign illness caused by myxoma virus in cottontail rabbits and the severe, regularly fatal disease it causes in domestic rabbits is the disease expression one induces with the agent in domestic rabbits that have recovered from infection with the fibroma virus (Shope, 1936b). Such animals are solidly immune to the fibroma virus and have circulating antibodies in their sera that neutralize that virus but not the myxoma virus However, despite the lack of myxoma virus-neutralizing antibodies, fibroma-recovered rabbits are much more resistant than normal ones to myxomatosis.

Injected by any route with myxoma virus, fibroma-recovered rabbits develop a local myxomatous lesion at the site of inoculation. The lesion develops progressively through the same stages as it does in normal rabbits. The local myxomatous tumor reaches its maximum size in about eight days and thereafter becomes firm and solid and finally gradually regresses, a process, of course, not to be observed in fully susceptible rabbits.

The course of myxomatosis in both fibroma-recovered and in susceptible rabbits is thus roughly similar for the first five or six days following infection, and only after this time are differences apparent The lesions in the fibroma-recovered animals remain localized and seldom are tumors to be observed other than at the site of injection. Also, the swellings of the eyelids and about the anal and genital orifices usually are absent. The local tumors caused by myxoma virus in fibroma-recovered rabbits, after first having the appearance of myxomas, gradually come to look more and more like the fibromas caused by fibroma virus in normal rabbits. However, the virus recovered from these fibroma-like lesions for as long as two weeks is

That neuromyxoma virus is definitely altered from its original myxoma state is indicated by the fact, first, that it produces a different histological picture from that produced by the classical myxoma virus (Hurst, 1937a); and second, that it does not yield a transforming substance capable of converting fibroma virus to myxoma in a Berry-Dedrick transformation (Hurst, 1937b). Furthermore, the neuromyxoma virus variant seemed to be stable, in that serial passage of it in domestic rabbits did not cause it to revert to classical myxoma virus. It would appear, therefore, that this agent is one belonging somewhere intermediate between classical fibroma and myxoma virus in the fibroma-myxoma virus complex, and that it bears a relationship to its susceptible hosts that is different from the host-virus relationships of either fibroma or myxoma virus.

The host reactions to classical myxoma virus have been studied in adult domestic rabbits, adult wild cottontail rabbits, and fibroma-recovered domestic rabbits; and in each type of host the disease expression induced by the virus is different. In the adult domestic rabbit, as already described, myxoma virus causes a disease characterized by the formation of myxomatous swellings at the site of inoculation and elsewhere over the body, and swellings of the eyelids, nose, and anal and genital orifices. It is practically invariably fatal

The reaction of the wild cottontail to myxoma virus is different from that of the domestic rabbit (Shope, 1936a). Hyperemia and thickening of the skin and subcutaneous tissue at the site of injection are sometimes observed six to twelve days after subcutaneous inoculation with the virus. Occasionally a small fibroma-like tumor eventually develops. The reaction resembles in no way the myxomatous lesion seen in domestic rabbits Furthermore, there is no evidence of a generalized reaction to myxoma virus in the cottontail; and, especially, no swellings of the eyelids, nose, or anal and genital orifices are to be seen. When myxoma virus is administered intratesticularly to cottontail rabbits, the reaction to the virus is limited to the injected testicle. After an incubation period of six to twelve days, the testicle becomes firm and enlarged and may remain in this condition for two weeks or longer. Examined at this time it is found to be inflamed and firm. It cuts as though fibromatous and contains white nodular areas The histological reaction consists of a mixture of inflammatory and young fibroblast-like cells, many of the latter forming compact whorls as often seen in fibroma virus infections. There is little to suggest the myxomatous reaction seen in domestic rabbits. After remaining firm and enlarged for two weeks or longer, the testicle gradually returns

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still characteristic myxoma virus capable of causing classical fatal myxomatosis when administered to susceptible rabbits.

Consideration of the relationships just described makes it apparent that the myxoma virus, like the fibroma virus, is capable of inducing differing disease expressions, and that these can be influenced by variations in either the virus or the host. Thus, neuromyxoma and classical myxoma virus, though immunologically apparently similar agents, differ markedly in the disease expressions they elicit in a common host, the domestic rabbit. *In like manner, the type of illness* caused in domestic rabbits, cottontail rabbits, or fibroma-recovered domestic rabbits by classical myxoma virus is determined seemingly not by the virus itself, but rather by factors within the hosts that alter the virus-host interaction in such a manner as to cause the virus to elicit three different disease expressions.

Summary

Both the host and the virus contribute to determining the final disease expression, and wide variations in the clinical picture can be effected by relatively minor variations in the character either of the host or of the virus. Age, species, and physiological and immune states have been shown to play a role in determining the host-virus relationship on the part of the host From the viral side, it has been shown that four closely related varieties comprising the fibroma-myxoma virus complex vary markedly in the disease expressions they individually elicit in various sorts of rabbit hosts.

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GENETIC BASIS OF CELL RESISTANCE

The Precancerous Nature of the Hyperplastic Alveolar Nodules Found in the Mammary Glands of Old Female C3H/Crgl Mice

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Since the original description of hyperplastic areas in the mammary glands of mice in 1906 by Apolant, many studies have been reported describing the behavior of these and related structures under a variety of experimental conditions. The presumed preneoplastic status of hyperplastic alveolar nodules has impressed several workers with the fact that these structures may provide valuable material for the study of the neoplastic change. The literature on this subject has been adequately reviewed (Bern, DeOme, Alfert, and Pitelka, 1958; Gardner, 1942; Huseby and Bittner, 1946; Muhlbock, 1956; Squartini, 1956)

Our opinion that hyperplastic alveolar nodules (Fig. 1) present favorable experimental material for studies concerned with the neoplastic transformation in the mouse mammary gland is based on the following 1) Their preneoplastic nature is supported by good indirect evidence, 2) they are present in large numbers in inbred mice, are recognizable as living entities, and therefore can be explanted into culture or transplanted from one host to another within the same strain, 3) they occur in a target organ which is subject to hormonal manipulation; 4) existing evidence suggests that the mammary tumor virus is an important inciting agent, thus permitting manipulation of another of the responsible agents.

The present report is limited to a study of the hyperplastic alveolar nodules found in the mammary glands of old, mammary tumor virus-infected, female C3H/Crgl mice. Experimental evidence will be presented concerning the origin, preneoplastic nature, transplantation behavior, and hormone requirements of these structures. In addition, our exploitation of this experimental material has led to several new technical procedures which may be useful to other workers.

The problem of selecting nodules and comparable samples of normal mammary gland tissue from living mice has been solved (DeOme, Bern, Berg, and Pissott, 1960). Considerable experience is required, but with practice they can be selected from untreated donors with an accuracy in excess of 95 per cent. The selection of nodules is greatly facilitated by the prior injection of cortisol, mammotropin, and somatotropin for a period of three to five days. For reasons presented later in this paper, the nodules become distended with milk, whereas normal mammary gland tissues are only slightly stimulated.

Attempts to compare nodules with mammary tumors and with normal mammary gland tissues by means of histologic, cytologic, and cytochemical methods, and by P^{32} uptake measurements, have demonstrated that the nodules resemble normal mammary gland tissues rather than mammary tumors (Bern, DeOme, Alfert, and Pitelka, 1958; DeOme, Bern, Berg, and Pissott, 1956; Harkness, Bern, Alfert, and Goldstein, 1957). These studies allow us to state that the typical nodule can be viewed in many respects as a lobule of prelactational tissue present in an otherwise inactive mammary gland (of either virgin or nonpregnant parous status).

Proof of the Preneoplastic Nature of Hyperplastic Alveolar Nodules

For the purpose of adequate experimental design, a class of structures may be defined as preneoplastic if it can be shown that it produces tumors more often and in less time than comparable samples of normal tissue. In our first attempts (Faulkin and DeOme, 1958; DeOme, Faulkin, Bern, and Blair, 1959) to demonstrate the preneoplastic nature of the nodule, we transplanted selected samples of normal mammary tissue and nodules from old C3H females into the dorsal subcutis of young C3H females. The samples could be identified and recovered for periods of time up to 80 weeks following transplantation. This experiment (Table 1) failed to demonstrate that tumors arose more frequently from nodules than from normal mam-

TABLE 1. Nodules and Normal Mammary Tissues Transplanted into the Dorsal Subcutis for Periods of 12 to 80 Weeks

Normal Transplants			Nodule Transplants		
No Transplanted	No Recovered and Viable	No of Tumors	No Transplanted	No Recovered and Viable	No of Tumors
81	41	0	80	60	1

mary tissues. Only one tumor developed from the 60 viable nodules, and no tumor arose from the 41 viable normal samples. These negative data could not be accepted as final since the transplants were placed in an unusual environment. Final determination of the preneoplastic status would depend upon the behavior of transplants placed into mammary fat pads where spontaneous tumors develop.

The use of the mammary fat pad as a transplantation site requires that the fat pad be free from host mammary gland tissue. The task of providing gland-free (cleared) fat pads was accomplished by the surgical removal of the mammary anlagen from the inguinal (#4) mammary fat pads of three-week-old C3H female mice before the gland elements had penetrated deeply into the fat pad. The surgical procedure involved has been described elsewhere (DeOme, Faulkin, Bern, and Blair, 1959). The undisturbed gland-free portion of the fat pad remains free from host mammary gland elements for the life of the animal.

In a typical experiment designed to test the preneoplastic nature of nodules, 19 nodules and 19 samples of normal mammary tissue were transplanted into the right and left cleared fat pads, respectively, of

TABLE 2. Mammary Tumors Derived from Nodule and Normal Transplants in Cleared Fat Pads

Tissues Transplanted	No Transplanted	No[1] Alive	No[2] Recovered	Tumors Present	Tumors Absent
Nodule	19	4	15	12*	3
Normal	19	4	15	3*	12

[1] Four host mice still living 56 weeks after transplantation
[2] Host mice were killed when palpable tumors were found
* One tumor was not palpable

The present report is limited to a study of the hyperplastic alveolar nodules found in the mammary glands of old, mammary tumor virus-infected, female C3H/Crgl mice. Experimental evidence will be presented concerning the origin, preneoplastic nature, transplantation behavior, and hormone requirements of these structures. In addition, our exploitation of this experimental material has led to several new technical procedures which may be useful to other workers.

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Attempts to compare nodules with mammary tumors and with normal mammary gland tissues by means of histologic, cytologic, and cytochemical methods, and by P^{32} uptake measurements, have demonstrated that the nodules resemble normal mammary gland tissues rather than mammary tumors (Bern, DeOme, Alfert, and Pitelka, 1958, DeOme, Bern, Berg, and Pissott, 1956; Harkness, Bern, Alfert, and Goldstein, 1957). These studies allow us to state that the typical nodule can be viewed in many respects as a lobule of prelactational tissue present in an otherwise inactive mammary gland (of either virgin or nonpregnant parous status).

Proof of the Preneoplastic Nature of Hyperplastic Alveolar Nodules

For the purpose of adequate experimental design, a class of structures may be defined as preneoplastic if it can be shown that it produces tumors more often and in less time than comparable samples of normal tissue. In our first attempts (Faulkin and DeOme, 1958; DeOme, Faulkin, Bern, and Blair, 1959) to demonstrate the preneoplastic nature of the nodule, we transplanted selected samples of normal mammary tissue and nodules from old C3H females into the dorsal subcutis of young C3H females. The samples could be identified and recovered for periods of time up to 80 weeks following transplantation. This experiment (Table 1) failed to demonstrate that tumors arose more frequently from nodules than from normal mam-

19 host mice (Table 2). The host mice were killed when palpable tumors appeared, and the location of the tumors was recorded.

Within a period of 56 weeks, eleven palpable tumors and one small tumor arose from nodule transplants, and two palpable and one small tumor arose from normal mammary gland transplants. The two palpable tumors derived from normal transplants appeared during the 24th and the 37th weeks after transplantation; whereas nine of the eleven palpable tumors derived from nodules developed before the 24th week (Table 3). Therefore, we feel justified in referring to hyperplastic alveolar nodules as preneoplastic structures, since mam-

TABLE 3 Distribution of the Appearance of Palpable Tumors by Number of Weeks after the Transplantation of Nodule and Normal Tissues into Fat Pads

Weeks after Transplantation	No of Tumors Arising from	
	Nodule Transplants	Normal Transplants
13	2	0
14	1	0
16	2	0
18	1	0
20	2	0
21	1	0
24	0	1
37	1	1
42	1	0

tissue transplanted into the cleared left #4 fat pad of a three-week-old female C3H mouse Compare with Figure 3 ×5

Figure 3 The normal #3 mammary gland of the virgin host mouse which developed the normal outgrowth shown in Figure 2 ×5

Figure 4 Typical hyperplastic alveolar nodules in the normal outgrowth derived from a sample of normal mammary gland tissue transplanted into the left #4 cleared fat pad of a three-week-old female C3H mouse ×15

Figure 5 Hyperactive outgrowth derived from a nodule transplanted into the right cleared #4 fat pad of a three-week-old C3H mouse Compare with the normal host gland in Figure 3, and with the normal outgrowth in Figure 2 ×5

Figure 6 An expanding nodule (arrow) derived from a nodule transplanted into a cleared #4 fat pad Compare with Figures 2, 3, and 5 ×9

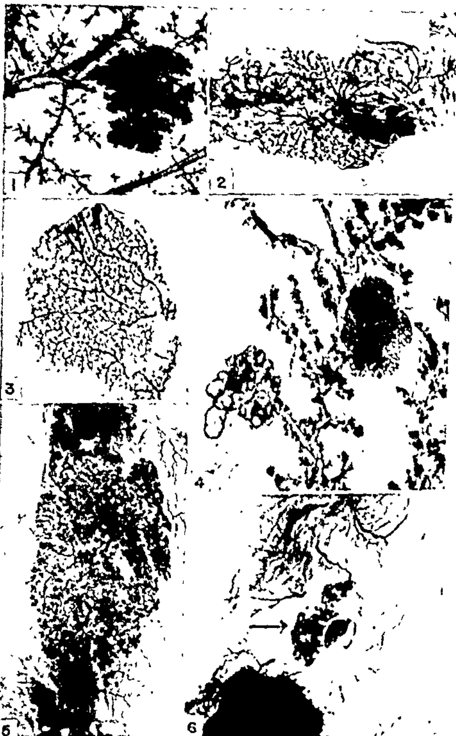


Figure 1 Typical hyperplastic alveolar nodules in the inactive mammary gland of an old nonpregnant but parous female C3H mouse ×15
Figure 2 Normal outgrowth produced by a sample of normal mammary gland

19 host mice (Table 2). The host mice were killed when palpable tumors appeared, and the location of the tumors was recorded.

Within a period of 56 weeks, eleven palpable tumors and one small tumor arose from nodule transplants, and two palpable and one small tumor arose from normal mammary gland transplants. The two palpable tumors derived from normal transplants appeared during the 24th and the 37th weeks after transplantation; whereas nine of the eleven palpable tumors derived from nodules developed before the 24th week (Table 3). Therefore, we feel justified in referring to hyperplastic alveolar nodules as preneoplastic structures, since mam-

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	Nodule Transplants	*Normal* Transplants
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14	1	0
16	2	0
18	1	0
20	2	0
21	1	0
24	0	1
37	1	1
42	1	0

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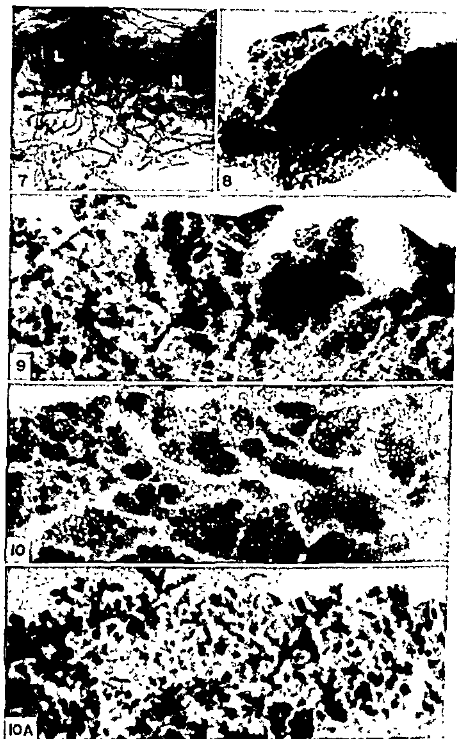


Figure 7 Normal-type outgrowth derived from a nodule transplanted into a

mary tumors arose from them more frequently and in less time than from comparable samples of normal tissue.

Behavior of Mammary Tissues Transplanted into Fat Pads

The nodules and normal tissues which had been transplanted into the dorsal subcutis were recoverable, and most of them retained their original structure with little change. However, when similar structures were transplanted into cleared fat pads they not only survived, but they grew and filled the fat pads to varying degrees within a period of eight to twelve weeks (DeOme, Faulkin, Bern, and Blair, 1959). The outgrowth patterns were typical of the transplanted tissues. Normal transplants produced normal outgrowths (Figs. 2 and 3) which, like the host's own mammary glands, responded normally to the endogenous hormones. Some of these normal transplants eventually produced hyperplastic nodules and mammary tumors. Among the normal outgrowths from normal transplants reported in Table 2, four showed hyperplastic nodules at points distant from the transplantation sites (Fig. 4), and two developed mammary tumors.

The outgrowths produced by hyperplastic alveolar nodules included a variety of patterns, most of which were easily distinguishable from normal. For descriptive purposes, the nodule-type outgrowths can be grouped into three general morphologic types. The most common type is a reasonable replica of a normal gland, but its elements are more densely packed together, and resemble the mammary gland of late pregnancy rather than the virgin host's glands (Fig. 5). A second type of hyperactive outgrowth is a slowly expanding, densely packed alveolar structure which we have designated as an expanding nodule (Fig 6). A third type of nodule outgrowth is a reasonable replica of the host's glands, and is composed of ductal elements, but it usually can be differentiated from a normal

cleared #4 fat pad Compare with Figures 2, 3, 5, and 6 L = lymph node, N = original nodule transplant ×5

Figure 8 Mammary tumor which arose within the hyperactive outgrowth derived from a nodule transplanted into a cleared #4 fat pad Compare with Figures 2 and 3 ×5.

Figure 9 Samples of the lactating (right) and nonlactating (left) portions, respectively, of this single outgrowth were transplanted into the right and left fat pads of each mouse through five successive transplant generations Compare with Figures 10, 10A and 11, 11A ×15

Figure 10, 10A Second generation transplants derived from lactating (10) and nonlactating (10A) portions of the hyperactive outgrowth shown in Figure 9 ×15



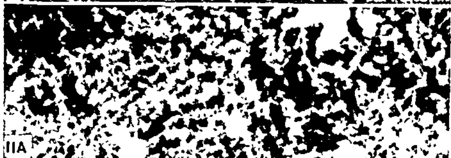






Figure 11, 11A Fourth generation transplants derived from the lactating

gland by minor structural irregularities (Fig. 7). Many variations and combinations of these outgrowth types are possible, either from a single nodule transplant or from different nodules in the same host animal

Mammary tumors may arise in the hyperactive outgrowth, but the outgrowth as a whole is not a tumor. Tumors appear at points distant from the transplantation site (Fig. 8), thus providing convincing proof that they need not arise directly from the nodule transplant. This observation suggests that the hyperactive outgrowth is especially prone to produce mammary tumors, and that cells having the ability to produce tumors equivalent to those demonstrated in the outgrowth must have existed within the nodule from which the outgrowth arose. The characteristic outgrowth patterns from both normal and nodule transplants provide an insight into the cellular composition of the transplants Normal outgrowths arise from normal cells in normal transplants, and hyperactive outgrowths arise from altered cells in nodule transplants.

The stability of some of the outgrowth patterns can be demonstrated by the serial transplantation of the morphologically recognizable types of outgrowth Two stable outgrowths were derived from a single nodule transplant. A portion of this outgrowth was lactating, and the remainder resembled the nonlactating gland of a mouse in the 12th to 14th day of pregnancy (Fig. 9). These two types of outgrowths were transplanted, respectively, into the right and left cleared fat pads of each host mouse through five successive transplant generations. In each of these generations, the lactating and the nonlactating character of the transplants were retained consistently (Figs 10 and 11). Similar results were obtained from the serial transplantation of an expanding nodule for five generations.

The nodules that occur spontaneously in intact glands do not form outgrowths, whereas similar nodules transplanted into cleared fat pads produce extensive outgrowths This observation has provided the basis for several preliminary experiments designed to explore the

(11) and prelactating (11A) portions of the hyperactive outgrowth shown in Figure 9 ×15

Figure 12 A nodule (N) transplanted into a fat pad already occupied by the host's own #4 mammary gland produced no outgrowth Compare with Figures 5 and 9 L = lymph node ×5

Figure 13 A cleared fat pad containing three outgrowths derived from three nodule transplants which were equally spaced along the length of the fat pad Arrows indicate the borders of the outgrowths ×7



Figure 11 Electron micrograph of parts of a lumen and of a bordering cell

factors controlling the growth pattern of the mammary gland. In one such experiment, hyperplastic alveolar nodules were transplanted into both the cleared and uncleared fat pads of adult mice. Outgrowths were produced in the cleared fat pads, whereas the nodules transplanted into the uncleared fat pads survived, but produced no outgrowths (Fig. 12). Similar results were obtained when normal tissues were used In another experiment, three nodule transplants were spaced equally along the length of a cleared fat pad. Each transplant produced an outgrowth, with the result that the fat pad was occupied by three small outgrowths. Each outgrowth occupied approximately one-third of the total fat pad, rather than the whole fat pad (Fig. 13). The outgrowths did not intermingle, and thus left a clearly visible, permanently unoccupied zone between them.

Both nodules and normal gland elements are subject to the growth-limiting effect of the presence of existing gland elements. However, mammary tumors will grow in either occupied or unoccupied fat pads and are said to be autonomous This sensitive measure of local autonomy should provide a useful tool by which certain questionable tumorlike outgrowths can be judged (Faulkin and DeOme, in press).

Electron Microscope Studies

In the course of our cytologic investigations the electron microscope was used in an attempt to discover differences in fine structure among mammary tumors (Fig. 14), hyperplastic alveolar nodules (Fig. 15), and normal mammary gland tissues (Pitelka, Bern, DeOme, Schooley, and Wellings, 1958) Viruslike particles were found in the lumina and in the cells of mammary tumors as had been previously described (Bernhard, Guérin, and Oberling, 1956; Dmochowski, 1956). In addition, all of the 23 nodules examined contained many particles in either the cytoplasm or the lumen or in both, but no particles were found in 31 of the 40 normal tissue samples examined (Fig. 16) Hyperactive outgrowths derived from the nodules transplanted into cleared fat pads were particularly rich in these

from a mammary tumor The lumen contains many viruslike particles ×32,000

Figure 15 Electron micrograph of parts of a lumen and of a bordering cell from a nodule The lumen contains secretory material and viruslike particles (arrow) ×32,000

Figure 16 Electron micrograph of a portion of a lumen and of a bordering cell from a normal prelactating lobule The lumen contains secretory material but no viruslike particles ×32,000

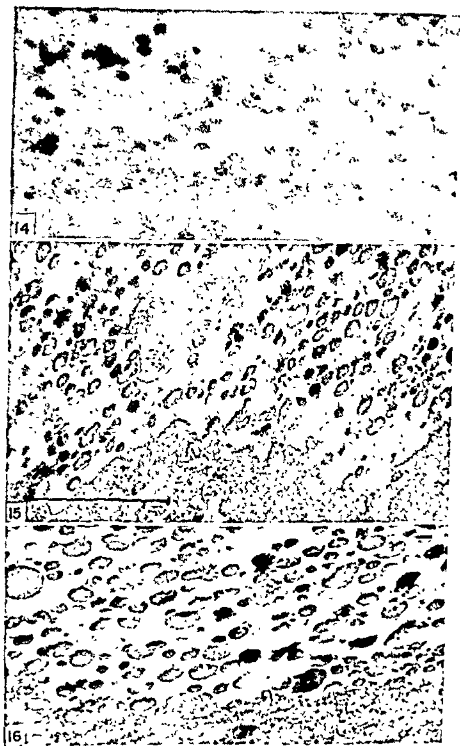


Figure 14 Electron micrograph of parts of a lumen and of a bordering cell

viruslike particles (Fig. 17). In these outgrowths the typically extracellular forms of the particles sometimes appeared clustered within a space bounded by a membrane, and surrounded by the intracellular forms of the particle This arrangement is suggestive of the aggregates reported by Dmochowski and Grey (1957) in mammary tumors. We have no evidence that particles arise in the Golgi region (Bernhard and Guérin, 1958).

ENDOCRINE STUDIES

The hyperplastic alveolar nodules which are found in the mammary glands of old virgin or parous C3H females resemble the normal lobules present during pregnancy. However, well-defined lobules are not usually found in the mammary glands of virgin females, and the lobules of pregnancy regress after weaning. These facts suggest that certain of the cells in the mammary gland are highly sensitive to hormones, which permits the formation and maintenance of nodules by means of only those hormones which are present in virgin or in nonpregnant, nonlactating, but parous females.

We have been attempting to determine the hormones necessary for the formation, maintenance, lactogenesis, and neoplastic transformation of the hyperplastic alveolar nodule. The purified hypophysial hormones used in these experiments were generously supplied by Professor C H Li Nodules were formed in young hypophysectomized-ovariectomized C3H mice treated with those hormonal combinations capable of inducing lobuloalveolar development in these animals (Nandi, Bern, and DeOme, 1960) These combinations included: estradiol (E) + progesterone (P) + somatotropin (STH); E + P + cortisol (F) + STH, E + P + mammotropin (MH) + STH, and E + P + desoxycorticosterone acetate (DCA) + MH. It is conceivable that any or all of these hormonal combinations could be responsible for nodule formation in parous females.

In virgin females neither P nor MH is believed to be present in sufficient amounts to induce nodule formation Accordingly, we searched for an effective combination that might occur normally in virgin mice We found that E + DCA + STH can induce both lobuloalveolar development and nodule formation in hypophysectomized-ovariectomized females when this treatment was continued for three

growth showing parts of several cells separated by cell membranes (CM), part of a nucleus (N), several mitochondria (M), and several clusters of viruslike particles (VP) in clear spaces bounded by membranes and surrounded by intracytoplasmic vesicular particles (IP) ×26,000

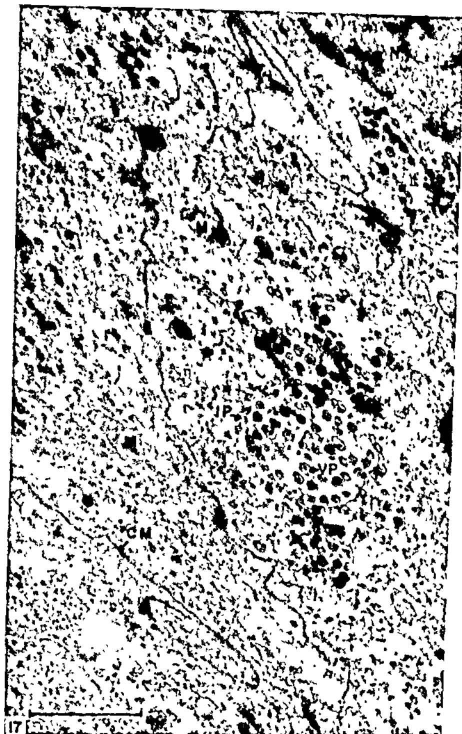


Figure 17 Electron micrograph of a portion of a typical hyperactive out-

months (Fig. 18). These hormones, or hormones with similar physiologic properties, are known to be present in normal virgin females.

The majority of hyperplastic alveolar nodules regress after hypophysectomy-ovariectomy-adrenalectomy (Fig. 19) (Bern, Nandi, and DeOme, 1957), but they can be maintained by appropriate hormonal treatments, such as either DCA or F plus either MH or STH (Fig. 20) (Nandi, Bern, and DeOme, 1960). MH probably is not present in appreciable quantities either in virgin or in multiparous nonpregnant females, but STH, which can replace MH as both mammogen and lactogen in C3H/Crgl mice (Nandi, 1958), is undoubtedly present (Solomon and Greep, 1958). DCA-like and F-like hormones also are found in mice (Hofmann, 1956). Thus, unlike the normal lobules, hyperplastic alveolar nodules can be maintained *in vivo* with minimum quantities of hormones such as are found in nonpregnant females.

Our studies of lactogenesis in nodules have shown that nodules and normal lobules differ with regard to their hormone sensitivity. This difference might be a result of either an alteration in the nodule cells which sensitizes them to hormones, or a circulatory or other structural change which may tend to concentrate hormones in the region of a nodule. Both *in vivo* and *in vitro* experiments have allowed us to examine these possibilities.

We found that 125 micrograms F + 100 micrograms of either STH or MH daily were ineffective in producing lactogenesis in normal lobules; whereas these combinations resulted in lactogenesis in some nodules (Fig. 21) (Nandi, Bern, and DeOme, 1960). When the dose of hypophysial hormone was increased to 1 mg./day, many of the normal alveoli also contained milk. In addition, a combination

10-week-old, hypophysectomized-ovariectomized female mouse which had received daily injections of β-estradiol (1 μg – 2 μg), desoxycorticosterone acetate (0.5 mg) and somatotropin (250 μg – 750 μg) for a period of three months ×10

Figure 19 A regressed nodule (arrow) and mammary gland from an old female mouse 21 days following hypophysectomy-ovariectomy-adrenalectomy ×15

Figure 20 Typical hyperplastic alveolar nodules in an old hypophysectomized-ovariectomized-adrenalectomized mouse treated with desoxycorticosterone acetate (0.25 mg) and somatotropin (1 mg) for a period of 21 days. Compare with Figure 1 ×9

Figure 21 Lactating nodules in an otherwise inactive mammary gland from a hypophysectomized-ovariectomized-adrenalectomized old mouse which had received daily injections of cortisol (125 μg) and somatotropin (100 μg). ×15







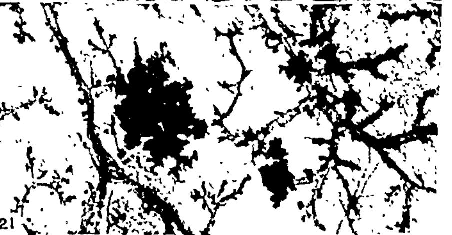


Figure 18 Lobuloalveolar development and nodule formation (arrow) in a

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Figure 21. Lactating nodules in an otherwise inactive mammary gland from a hypophysectomized-ovariectomized-adrenalectomized old mouse which had received daily injections of cortisol (125 μg.) and somatotropin (100 μg.). ×15.

TABLE 4. Behavior of Normal and Nodule Explants in Organ Culture in Hormone-Supplemented Medium "199"

Hormone Supplement[1] µg/ml		Explants[2]			
		Normal		Nodule	
F	MH	M	S	M	S
8	0	—	—	—	—
8	31	+	—	++	++
8	62	+	—	++	++
8	125	++	++	++	++
8	225	++	++	++	++
8	450	++	++	++	++

[1] *F* = Cortisol, *MH* = Ovine mammotropin

[2] *M* = Maintenance, *S* = Secretory stimulation, — = Degeneration of explant or lack of secretory stimulation, + = Partial maintenance, ++ = Good maintenance or stimulation

such as DCA + STH resulted in lactogenesis in a small number of nodules, but was ineffective in the induction of lactogenesis in normal lobules (Bern, Nandi, and Finster, 1959). These experiments suggest that at least some of the nodules are more sensitive than normal lobules to a lactogenic combination of hormones, and that some of the nodules even differ qualitatively in regard to the hormones that can induce lactation.

Maintenance and lactogenesis occurred in prelactating lobules and in nodules in organ culture when 8 micrograms of F and graded doses of MH were added to the synthetic medium "199" (Table 4). Under these conditions the nodules responded to much lower doses of MH than did the normal lobules (Elias and Rivera, 1959). Several preparations of MH were used. Only one of these resulted in the maintenance and stimulation of prelactating lobules, but the nodules responded to all the MH preparations tested. These *in vitro* experiments eliminate the possibility that the altered sensitivity of the nodule is the result of circulatory or other environmental changes.

It is probable that the hormone-sensitive population of cells comprising a nodule arose from a few altered cells located in the terminal ducts. Indirect evidence to support this view is available. Nodules placed in organ culture in unsupplemented synthetic medium "199" for a period of five days undergo alveolar but not ductal degeneration and then will produce nodule-type outgrowths when transplanted

TABLE 5 The Effect of Various Hormonal Manipulations on Hyperplastic Alveolar Nodules Transplanted into the Mammary Gland-free Right and Left #4 Fat Pads of Female C3H Mice

Age at Transplantation (weeks)	Operations Performed[1]	Hormones Injected[2]	Age at Termination (weeks)	No. of Nodules Transplanted	No. of Nodules Recovered and Viable	No. of Nodules Producing Tumors
3	$\overline{O}$	none	13–17	48	45	12
3	$\overline{A}$	none	13–17	26	24	12
3	$\overline{O} + \overline{A}$	none	17	10	10	none
3	$\overline{H}$	none	17	28	28	none
10	$\overline{H} + \overline{O}$	none	23	6	6	none
10	$\overline{H} + \overline{O}$	E+P+STH	18–23	8	8	3
10	$\overline{H} + \overline{O}$	DCA+STH	23	8	7	3
10	$\overline{H} + \overline{O}$	E+DCA+STH	18–23	10	10	2

[1]Operations were performed two weeks following nodule transplantation.
Abbreviations used are as follows: $\overline{O}$, ovariectomy; $\overline{A}$, adrenalectomy; $\overline{H}$, hypophysectomy.

[2]Daily hormonal treatments were started one day following the operations listed.
Abbreviations used are as follows: E, β-estradiol; P, progesterone; DCA, desoxycorticosterone acetate; STH, bovine somatotropin.
The following daily doses were used. E = 1–2 μg.; P = 1 mg.; DCA = 0.5 mg.; STH = 250–750 μg.

into cleared fat pads. Normal lobules cultured in a similar manner produce normal outgrowths. This experiment shows that cells possessing altered hormone sensitivity exist in the ducts of a nodule, and supports the view that the altered alveoli of nodules arose from such altered duct cells. The hormone-sensitive duct cells would appear to have a selective advantage within the fat pad since they would be stimulated to undergo lobuloalveolar development in response to normal endogenous hormones.

Preliminary experiments suggest that hormones may be involved in the neoplastic change which occurs in nodules and in their outgrowths (Nandi and Bern, 1959). For example, lactating nodules selected from old C3H females which had received F + STH + MH for three days were transplanted into the cleared fat pads of young C3H females. After 14 days, the host animals were subjected to ovariectomy, adrenalectomy, and hypophysectomy (Table 5). Within eight to twelve weeks, tumors appeared in the ovariectomized group and in the adrenalectomized group, but no tumors were found in the ovariectomized-adrenalectomized or in the hypophysectomized groups. This experiment suggests that the pituitary and either the adrenal or the ovary may be required for the neoplastic transformation.

In order to test this possibility, hosts which had been hypophysectomized-ovariectomized 14 days after the transplantation of the nodules were treated with the following hormonal combinations: E + P + STH; E + DCA + STH; and DCA + STH. Tumors were found in some mice in all of these groups within eight to twelve weeks, thus supporting the idea that the neoplastic transformation requires an ovarian or adrenal steroid plus a pituitary hormone. The minimum hormonal requirements for the neoplastic transformation of nodules remain to be determined, but we would emphasize at this date that one tumorigenic combination (DCA + STH) does not involve estrogen, which is generally considered to be an essential hormone for mammary tumor induction in mice.

The limited data presented herein do not suggest the manner in which the hormonal state of the host influences the neoplastic transformation. Further studies will help decide whether the essential hormonal factors are *inductive* or *permissive* in tumorigenesis.

Conclusion

On the basis of the evidence presented herein it is possible to construct a working hypothesis which accounts for the origin, develop-

ment, maintenance, and cell composition of the preneoplastic hyperplastic alveolar nodule. This hypothesis must be limited to mammary tumor virus-infected *C3H/Crgl mice*, since comparable evidence concerning other strains is not available.

It is tempting to assume that the viruslike particles are identical with the biologically active mammary tumor virus. Such an assumption would permit the conclusion that the hyperplastic alveolar nodule is a virus lesion, and that its precancerous nature is the result of interaction between the virus and the mammary gland cells. It is probable that the virus produces alterations in certain mammary duct cells The alterations considered here are those which produce an increased sensitivity to the endogenous hormonal combination which stimulates lobuloalveolar development. In virgin or nonpregnant parous C3H females, this combination need not include either mammotropin or progesterone. The hormone-sensitive duct cells would have a selective advantage in the normally occupied fat pad and therefore would produce a sharply circumscribed lobule-like structure composed of hormone-sensitive cells. This lobule-like structure in an otherwise resting gland is the hyperplastic alveolar nodule. The nodule persists in both virgin and parous C3H females because the hormones necessary for its continued maintenance are present. The increased hormone sensitivity of the nodule is a characteristic of the cells of the nodule and is not due to local environmental factors.

Several types of alterations have been demonstrated in the nodule cell population. These produce a variety of outgrowth patterns and functional states in cleared fat pads; some of these outgrowth patterns are stable for at least five transplant generations. It can be postulated that the stable outgrowth patterns represent populations of cells which have been permanently altered as a result of virus-host cell interaction. Nodules occurring in normally occupied fat pads do not produce outgrowths, and thus do not reveal their cellular composition, owing to the restraining influence of the adjacent mammary gland elements.

The transplantation studies presented herein suggest that the appearance of a nodule represents the growth of an altered population of cells, and that the appearance of a tumor represents the subsequent occurrence and growth of another altered population of cells. This hypothesis provides a method by which one can visualize the production of sequential and apparently stable alterations in normal cell populations *in vivo*.

This working hypothesis must be considered with due regard for

into cleared fat pads. Normal lobules cultured in a similar manner produce normal outgrowths. This experiment shows that cells possessing altered hormone sensitivity exist in the ducts of a nodule, and supports the view that the altered alveoli of nodules arose from such altered duct cells. The hormone-sensitive duct cells would appear to have a selective advantage within the fat pad since they would be stimulated to undergo lobuloalveolar development in response to normal endogenous hormones.

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ACKNOWLEDGMENTS

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Tumor Cell Resistance to Antimetabolites and Its Possible Genetic Implications

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The treatment of the cancer patient today involves surgery, irradiation, and chemotherapy. Only the latter is considered in this report. The objective of cancer chemotherapy is to secure control and cause regression of cancer with chemicals possessing greater toxicity to the tumor cells than to the normal host cells or host cell systems. In the light of present knowledge, it is believed that the therapy must be directed toward cancer cell perpetuating and growth factors rather than toward causative mechanisms. There are many types of theories of perpetuating factors in cancer, perhaps best termed adaptive biochemical changes. A complicating factor is that many carcinomas have the structure of neoplastic cells in a matrix of connective tissue, and thus growth and chemotherapy must be evaluated in terms of an increase or decrease in a number of various structural units. The complicating factors seem to be simplified when cancer is grown in the ascitic form and are perhaps also simplified in the case of leukemias.

A tragic aspect of the chemotherapeutic approach to cancer is that eventually, and, at present, inevitably, clinical control of the disease is blocked. Reasons for failure are: (1) Initial resistance of tumor cells to therapy; (2) differential recovery of damaged normal tissue, such as bone marrow cells, as compared with tumor cells, thus preventing continued or repeated therapy; and (3) development of tumor cell resistance to the antimetabolite, analogue, or drug used in

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have reported their experience in the treatment of patients with choriocarcinoma and related trophoblastic tumors with a *folic acid antagonist* (methotrexate). The patients were treated according to a highly intensive regimen. It is reported that methotrexate resistance, when it has occurred, has assumed a fixed pattern in most instances. In some patients, some recovery of responsiveness has been noted after varied intervals of therapy.

The folic acid antagonists have been studied in many strains of transplanted leukemia in mice. Responses have varied from no effect in chloroleukemia AK 1394 and myeloid line X in the F strain, lymphomas 1 and 2 in strain A mice, and L 1358 in DBF_1 mice; to a doubling or tripling of survival time in AK 4 leukemia, marked inhibition of growth of subcutaneous tumor masses, and increase in survival time of L 1210 in DBA mice; to frequent complete cures in Line I in C58 mice (Burchenal, 1954).

Potter (1958) has described the attainment of resistance to amethopterin used in the treatment of an experimental near-tetraploid lymphocytic neoplasm, P288. Survival data indicated that in almost all cases there was more than 100 per cent increase in survival time of tumor host mice. Treatment was somewhat limited by host toxicity, and sustained extended treatment was not possible. No mouse given 10^5 or more cells and subsequently treated with amethopterin survived indefinitely.

In attempts to develop amethopterin-resistant lines of P288, multiple transfers through amethopterin-treated mice were required Cell populations that develop in mice in the course of a single transfer generation do not develop full resistance to amethopterin. After 19 transfer generations, a heritable resistant line was developed. After 14 transfers through untreated hosts, resistance has remained. A similar study indicates that resistance is attained progressively and not suddenly.

An amethopterin-dependent subline of leukemia L 1210 has been produced (Law, 1956). This subline is dependent upon amethopterin for optimal growth. The possibility was considered that resistance developed to folic acid allowed increased or decreased sensitivity to other antileukemic *agents An unequivocal cross-resistance* was obtained when the dependent cells were grown in mice receiving four closely related antifolic compounds. 8-azaguanine resistant and 6-MP resistant L 1210 leukemias are known to be considerably more sensitive to amethopterin than the original line of leukemia from which the resistant lines were derived. In a similar study (Hutchison,

the therapy. An understanding and solution of the problem of antimetabolite and drug resistance of cancer, the part of the problem under consideration, is worthy of the best efforts of investigators. The purpose of this report is to review this problem from the standpoint of genetics, since it appears that attainment of resistance may be related to a process of selection on the cellular level, whereby the favored cells are those which have acquired the capacity of escaping control by antimetabolites. The persistence of resistance also leads one to consider genetic mechanisms.

Tumor Cell Resistance

The term "resistance" is applied to the temporary or permanent capacity of a cell and its progeny to remain viable and multiply under environmental conditions that would destroy or inhibit other cells.

Tumor cell resistance to drug therapy following initial sensitivity has been observed with a variety of agents: amethopterin, azaserine, cortisone, and 6-mercaptopurine, to give a few examples. How do these resistant cell lines arise? The problem is not unique. In a related field, microbiological drug resistance has received much attention. A single explanation for resistance is not easy to secure in either field unless it be in very general terms, since the drugs in question have apparently unrelated modes of action. One or two examples of antimetabolite resistance by cancer cells will be given.

Folic acid, and citrovorum factor, its biologically more active counterpart, are vitamins necessary for the growth and maturation of normal erythroid and myeloid tissue of the marrow. Fortunately, from the point of view of the chemotherapist, they are even more necessary for the growth of some of the cells of some forms of acute leukemia. The folic acid antagonists, aminopterin and amethopterin, have thus been used successfully in the treatment of children with acute leukemia. The antagonists differ from folic acid mainly in the substitution of an amino group for a hydroxyl group in the 4 position of the pteridine ring. Treatment with either of these factors causes a relative deficiency of citrovorum factor throughout the body. This deficiency specifically damages certain types of cells, particularly, in the case of cancer, those of acute leukemia. It is important to note that in an extensive study, only 37 per cent of the children with acute leukemia responded to treatment, while many with what appeared to be morphologically identical disease gave no indication of response to the folic acid antagonists (Burchenal and Holmberg, 1958) Investigators (Li, Hertz, and Spencer, 1956; Hertz *et al.*, 1958) also

have reported their experience in the treatment of patients with choriocarcinoma and related trophoblastic tumors with a folic acid antagonist (methotrexate). The patients were treated according to a highly intensive regimen. It is reported that methotrexate resistance, when it has occurred, has assumed a fixed pattern in most instances. In some patients, some recovery of responsiveness has been noted after varied intervals of therapy.

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1958) it was noted that three lines of L 1210 leukemia resistant to amethopterin were also resistant to 6-MP. Even though azaserine produces only a slight effect against L 1210 leukemia, a resistant line was selected. The azaserine-resistant L 1210, line XII, was resistant to amethopterin and 6-MP, and had slightly increased sensitivity to 5-fluorouracil.

Antimetabolites and Cancer Chemotherapy

A metabolite, by definition, is a product of metabolism. An antimetabolite is any substance which interferes with a metabolite, especially with the function of a metabolite. The basic concepts have arisen from studies of bacterial nutrition, of animal nutrition, of enzyme action and composition, and of pharmacological antagonism of drugs (Woolley, 1952).

From a biochemical standpoint, we may consider an antimetabolite to be a closely related analogue of a normal metabolite which has been shown to act on a well-defined enzyme system or metabolic process, and which has its action reversed by the particular metabolite involved.

The detection and measurement of antimetabolites may be accomplished by the study of any of a variety of inhibiting effects, such as that upon growth, respiration, or fermentation, as well as upon the function of a specific enzyme system. Antimetabolites also may be versatile disorganizers of many biological functions and have, under certain circumstances, strong mutagenic as well as antineoplastic qualities (Hemmerly and Demerec, 1955). The amino acid analogue, azaserine, is an example of such an antimetabolite.

Among the antimetabolites of theoretical or useful interest in cancer we might mention (1) the purine analogues, 6-mercaptopurine and 8-azaguanine; (2) the pyrimidine analogues, 6-azauracil and 5-fluorouracil; (3) the vitamin analogues, amethopterin and aminopterin; and (4) the amino acid analogues, azaserine and 6-diazo-5-oxo-L-norlucine (DON).

Figure 1 is intended to show, diagrammatically, the relation of genes to antimetabolite influences on cancer cells. These are presumably operative relationships in normal cellular susceptibility and resistance, as well as in the attainment, reversal, and persistence of resistance. Gene action is interpreted here as operating through determination of macromolecular pattern.

It is convenient to think of no sharp cleavage between genetic and environmental (antimetabolite, in this instance) determination, but

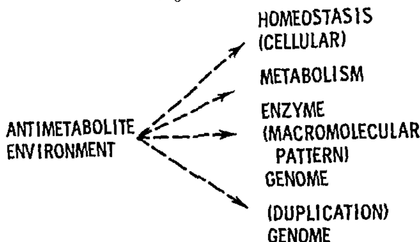


Figure 1 Diagram of relation of genome and antimetabolite environment to cellular characters of cancer at levels of increasing organization.

rather of a probability distribution of physiological states, due to yet uncontrolled factors. The term penetrance (Timofeef-Ressovsky, 1927) might be employed to describe this condition. A simple hypothesis is that the antimetabolite resistance reflects overlapping aspects of a single varying macromolecular pattern imposed on the cellular substrate by a corresponding pattern in the gene. Homeostatic, as used, refers to the ability of the cell to react adaptively to environmental change. A genotype that would not allow the cells to function under antimetabolic interference would not yield a homeostatic mechanism.

Patterns of Evolution for Attainment of Cancer Antimetabolite Resistance

Gene mutation, chromosome change, restriction of the population size, natural selection, and development of isolating mechanisms are the common denominators of almost all evolutionary histories (Dobzhansky, 1951). The nature of cancer cell reproduction creates very special problems which must be considered Pure lines and clones of somatic cells do not exchange genes, since such an exchange is precluded by their very method of reproduction. The genotype of each cell line is a closed system, isolated from similar systems, and capable of changing only through mutation. In Wright's (1956) symbolic picture of "adaptive peaks" and "adaptive valleys," the peaks, it

will be remembered, symbolize groups of related gene combinations which make their carriers fit to survive and reproduce in a given environmental niche; the valleys symbolize the gene combinations which are low in the existing environments. Groups of cancer cells may be thought of as living populations, the cells of which are almost surely clustered around some "adaptive peak" in the field of gene combinations, while the "adaptive valleys" remain more or less uninhabited. Cell clusters will exist which, in a way, are analogous to those encountered in sexual forms.

The environmental change produced by man's interference with the habits of the cells, through the use of antimetabolites in this specific case, lowers the adaptive values of some of the genotypes which were favored before the change, and augments the value of other genotypes which were discriminated against in the old environments. Cell lines may become extinct if no new genetic elements (mutations) necessary to produce the new adaptive genotypes are available, or if the requisite constellations of these elements do not appear in time. In order to survive, the cell must reconstruct the gene pool of its component population and arrive at the gene combinations that represent the new adaptive peaks. Abundant and consistent evidence exists that antimetabolites aid in the development of a basis for genetic variation-mutation: chromosome breakage, spindle interference, change in chromosome number, etc.

The existence of unoccupied adaptive peaks is a consequence of the fact that innumerable gene combinations have never been formed and tried out. This is obviously true, because the number of possible combinations far exceeds the number of cell lines that a tumor population may have. The possibility of adaptation of cancer cells against antimetabolite therapy would not be known without trial.

An increase in the stringency of selection will force the cells to withdraw to the highest level of their adaptive peak. The variability is reduced, leading to an extreme specialization which in nature may prove fatal to the cell line in the event of environmental change. New collateral sensitivities may become operative at this time. Examples of this have been noted. Another way of stating this is that selection favors those variants useful at a given time, regardless of their eventual value. Types may be perpetuated which are not well adapted when a second and different antimetabolite is used for growth interference.

Figure 2 illustrates one of two different interpretations for attainment of drug resistance, as suggested by recent studies in the chemo-

Mechanism of Emergence of Drug Resistant Cells

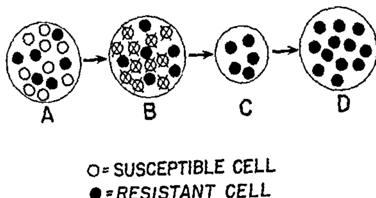


Figure 2 Method 1 selection—no changed cell required *A*, original cell population, *B*, action of selective environment, *C*, restricted cell population; *D*, outgrowth of resistant cell type Diagram illustrates a method of selection when there is a large number of mutant types in the original tumor

therapy of cancer. Although the diagram is elementary in form, it undoubtedly is basic. It appears that the changes to resistance and susceptibility occur spontaneously and rather generally among populations of leukemic cells, and the role of the antimetabolite is merely that of a selective agent. Increase in resistance has been seen to occur gradually, perhaps stepwise, over a period of time. In this regard, Law (1952) obtained evidence through use of a "fluctuation test" that the variant resistant and susceptible cell types arise as mutants during normal growth, the unfavorable environment being merely selective in their isolation

Figure 3 illustrates a second postulated method for cell selection in an unfavorable environment. In this instance, the unfavorable environment is assumed to induce changes in some of the cells—changes which aid in the selection of resistant cell types. The known mutation-producing properties of antimetabolites, together with other chromosomal and cellular disruptive effects, would tend to favor this hypothesis.

By either of these two interpretations, one might be referring to some of the postulated or actual cellular changes associated with antimetabolite resistance; for example: (1) the appearance of an en-

will be remembered, symbolize groups of related gene combinations which make their carriers fit to survive and reproduce in a given environmental niche; the valleys symbolize the gene combinations which are low in the existing environments. Groups of cancer cells may be thought of as living populations, the cells of which are almost surely clustered around some "adaptive peak" in the field of gene combinations, while the "adaptive valleys" remain more or less uninhabited. Cell clusters will exist which, in a way, are analogous to those encountered in sexual forms.

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Figure 2 illustrates one of two different interpretations for attainment of drug resistance, as suggested by recent studies in the chemo-

In discussing this work, one must keep in mind that the concept of mutation ranges from point mutations, through chromosome breakage, to ploidy. It also is interpreted that mutagens can act either directly on the gene or indirectly; indirect action being accomplished through interfering with the mutagen-antimutagen balance. Alongside the slow method of point mutation, there exists a method of rapid emergence of cell variants by polyploidy. In the wide sense of the term "mutation," polyploidy (euploidy and aneuploidy) is a mutational change. The importance of polyploidy in evolution of the cancer cell is believed to be considerable. Doubling or increasing the chromosome complement often produces physiological changes which are comparable to those produced by gene mutations. The cell size in polyploids is usually greater; the water content, osmotic pressure, vitamin content, immunological characteristics, etc., may be changed.

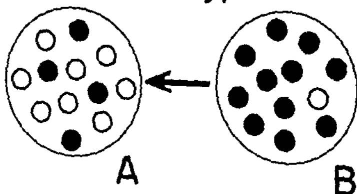


Figure 4 Diagram showing possible "backdrift" of antimetabolite-resistant cancer cell population to susceptible cell, due to failure to "fix" or select completely new cell type before treatment is withdrawn. *A*, incompetely selected resistant cell population; *B*, susceptible cell population.

zyme to break down the chemotherapeutic agent; (2) the development of alternate metabolic pathways (lack of ability of 6-MP and 8-azaguanine to convert these antagonists to ribotides) (Brockman, Wallace, Sparks, and Simpson, 1957); (3) decrease in membrane

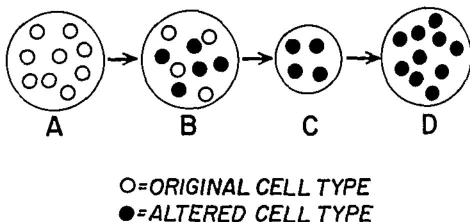


Figure 3 Method 2 selection—changed cell required. *A*, original cell population; *B*, action of selective environment causing altered cell type; *C*, restricted cell population; *D*, outgrowth of resistant cell type. Diagram illustrates a method of selection when antimetabolite, through mutation and cell disruptive influences, creates cell type for selection.

permeability and modification of transport; (4) change in relative affinity of enzymes; and (5) change in the way the host handles the antimetabolite, including transformation phenomena.

One might digress for a moment for a more general look at the chemical mutagen problem. Attempts to enhance the mutation rate of plants and animals are almost as old as the modern science of genetics (McDougal in 1911 and Morgan in 1910: see Westergaard, 1957). The real breakthrough came at the end of the Second World War; in 1946 Rapoport demonstrated the mutagenic effect of formaldehyde on Drosophila; in 1943 Oehlkers injected ethylurethane into the flower buds of Oenothera and thereby induced chromosome mutations, and Auerbach showed that a war gas, "mustard gas," greatly enhanced the mutation rate in Drosophila (Auerbach, 1957). These observations led to many discoveries.

genes and inherited in a normal Mendelian manner. The simplest interpretation of these findings is that the cytoplasm of every stock contains discrete bodies of several kinds, plasmagenes, which reproduce themselves provided the nuclear genotype is favorable for their perpetuation.

In experimental leukemias, such as the Gross and Friend leukemias, self-reproducing viruslike particulates, apart from chromosomal material, apparently multiply within the cell and can be isolated. In leukemias of this type, therapy and resistance must be considered on a cell-particulate level Changes of such particles may or may not be due to alteration of nuclear genes, or possibly to qualitative changes in the viral agents themselves. Antimetabolite resistance in these leukemias has not been demonstrated up to this time.

Transmissible changes in cells reproducing vegetatively warrant discussion, because somatic cells in which malignant transformation occurs do not undergo sexual reproduction The most widely quoted example of directed change is transformation of pneumococcal types; this has been extended to several other species as well. Griffith (1928) found that he could transform a nonencapsulated R variant into encapsulated cells of virulent smooth type by injecting line R bacteria into mice, along with heat-killed S cells which had a serological type different from that of the S strain from which the R cells had been derived originally. Avery, MacLeod, and McCarty (1944) found that the transforming principle is deoxyribonucleic acid. The change is a permanent one, and active DNA can be isolated from the transformed organism. Insofar as is known to the author, conclusive evidence for related transformation within cancer cells, in relation to antimetabolite resistance, has not been presented.

Cell Clones

Much information regarding changes in growth of somatic and cancer cells may be obtained from the *in vitro* study of cells descended from a single cell. Clonal studies on normal cells and on their neoplastic transformation *in vitro* have been reported by Sanford (1958). Since a clone originates from one cell, the occurrence of a new cell-type must result from a change or transformation in the cells.

The study was initiated by culturing a strain of cells from subcutaneous adipose tissue taken from the lateral fat pad of a mouse. After three months of culture, one cell was isolated and grown to a large culture by a method where one cell is completely isolated from all

The production of polyploids is the most powerful tool yet available to geneticists for molding living matter into new shapes. Polyploidy may be considered a short cut by which the cells may adapt themselves easily to a changing environment. Often it produces innumerable variations on old themes, but does not originate really new departures. Following the occurrence of polyploidy, the genes lying in different chromosomes will mutate in different directions. Polyploidy is thus a mechanism whereby both gene number and gene variety are increased. Hauschka (1957), Levan and Biesele (1958), Koller (1956), and others have described the great variability in chromosome number in cancer. The ascitic form of cancer has been especially variable. Reproductive isolation of cell lines preserves the integrity of the antimetabolite-adapted cells.

Figure 4 is a simple diagram illustrating the "backdrift" of resistant to susceptible cell population when "fixation" or selection has not been complete. It is assumed that, if proper cell types are retained in the population, lack of antimetabolite-selection pressure will allow a return to the original balance of cell types or to essentially the original cell population. It is possible to suggest that continued absence of the antimetabolite from the environment for extended periods of time will allow chromosomal and other mechanisms within the cell to adjust to the non-antimetabolitic environment in a manner somewhat similar to that outlined in Figure 3, Method 2 for selection for resistance. Antimetabolite dependence for optimal growth as reported by Law (1951) may be an extreme case, perhaps not altogether unexpected, in which this outlined mechanism would operate to require the antimetabolite.

Cytoplasmic Resistance Mechanisms

A number of cases have been described where abnormal cytoplasmic elements, plastomes, plastids, and other self-reproducing cell particulates are associated elements of the genome. A true-breeding high sensitivity to carbon dioxide in Drosophila appears to be dependent on a self-perpetuating infective substance transmitted primarily through the egg cytoplasm The plasmagene system of *Paramecium aurelia* transmitted in the cytoplasm also should be considered in this regard. Different stocks vary in serotype potentialities. Crossing representatives of the serotypes reveals that the serotype differences within a stock are inherited through the cytoplasm, and that their nuclear genes are alike. However, the potentiality of being able to produce a certain variety of serotype is determined by the

genes and inherited in a normal Mendelian manner. The simplest interpretation of these findings is that the cytoplasm of every stock contains discrete bodies of several kinds, plasmagenes, which reproduce themselves provided the nuclear genotype is favorable for their perpetuation.

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The study was initiated by culturing a strain of cells from subcutaneous adipose tissue taken from the lateral fat pad of a mouse. After three months of culture, one cell was isolated and grown to a large culture by a method where one cell is completely isolated from all

other cells in a separate flask. The isolated cell was grown into a clone culture, which was divided during subsequent transplantation to establish a total of 17 independent cell lines.

The clones eventually exhibited a number of characteristic differences—sarcoma-producing capacity when explanted, metabolic pattern, enzymatic pattern, responsiveness to growth restraints, chromosomal constitution, and probably antigenicity.

It would be of interest to investigate the reaction to antimetabolites in this clonal series and study possible cell line resistance in relation to chromosome and other variation.

Seven pure cell lines derived from leukemia L 5178 were obtained by the clonal growth of single cells (Fischer and Jaffe, 1958). The pure lines were uniformly sensitive to amethopterin *in vitro.* When the pure lines were cultured in gradually increasing concentrations of amethopterin for periods up to 120 days, growth response indicated the gradual emergence of resistant populations. The degree of resistance of most of these selected lines remained constant in culture in the absence of amethopterin. A resistant population selected in mice and cultured in the presence of amethopterin demonstrated a reduction in amethopterin sensitivity. A moderately resistant line from tissue culture exhibited full sensitivity when grown in mice. We await full publication of these experimental results.

Conclusion

A genetic interpretation for resistance of cancer to antimetabolite therapy appears to offer no conflict between known genetic mechanisms and observed facts pertaining to resistance, attainment of resistance, reversion to susceptibility, fixation of resistance, and collateral sensitivity. A well-documented fact is that chromosomes tend to "loosen up" in tumor cells. This may form an important mechanism in the attainment of antimetabolite and drug resistance.

Tissue culture studies of single cells and of cell clones have shown that these cells, which resemble somatic cells, change through a number of cell generations in which the environment ostensibly remains constant.

There is evidence from chromosome variation, mutation effects, and many other known antimetabolite effects that a mechanism exists for change in genetic constitution and for the operation of selection under drug (antimetabolite) pressure.

It is evident that, from a genetic standpoint, evolutionary adaptation is the central theme for explanation of tumor resistance to anti-

metabolites. Genetic variability is the raw material for selection. The variability may include minute mutations, chromosome aberrations of all sorts, and, in general, material ranging from genes to genome. The genetic material must be considered in relation to cell environment: cell particulates, cells as a whole, and cells as individuals in a population. If small differences in the rate of reproduction exist between tumor cell groups due to the use of antimetabolites, the role of selection in attainment of resistance is an obviously important one.

ACKNOWLEDGMENTS

The work reported in this paper was supported in part by Research Grant CY-3784 from the National Cancer Institute, Public Health Service, and Contracts SA-43-ph-1923 and SA-43-ph 2445 from the Cancer Chemotherapy National Service Center, Bethesda, Maryland, and Grant T-47 from the American Cancer Society, New York, N. Y.

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could influence any interpretation of breed susceptibility. The animals included in this study were considered to be selected randomly since each herd, and animal within a herd, had about an equal probability of being included. The data with regard to eye health have come primarily from: (1) direct observations of eyes and lesions (where a clinical observation and a colored photograph of each eye were made, followed by biopsies of 20 per cent of the lesions); and (2) disposal data, which indicate whether an animal was removed from a herd because of cancer eye.

The pathological development of ocular carcinoma is apparently a continuous process wherein lesions on the eyeball, nictitating membrane, and caruncle proceed from a so-called plaque, sometimes through a papilloma stage, to carcinoma; and those on the lids proceed from areas of keratoses and/or acanthoses with focal ulceration, sometimes through a papilloma stage, to carcinoma (Russell, Wynne, and Loquvam, 1956). The clinical diagnoses of these lesions compared with microscopic ones, without regard to the type of lesion, show a specificity of 99 per cent with 1 per cent false positives (Wynne, 1958). The specificity of diagnoses from disposal data is lower, but probably at least 80 per cent, and more likely than not, 90 per cent.

TABLE 1. Age Distribution of Cancer Eye

Age last observation (years)	Survivors to each age	Total cancer eye	Frequency per 1000
3	4960	4	0.8
4	4238	8	1.9
5	3668	29	7.9
6	3024	24	7.9
7	2504	45	18.0
8	2069	40	19.3
9	1659	48	28.9
10	1314	43	32.7
11	926	25	27.0
12	407	5	12.3
13	171	5	29.2
14	80	3	37.5
15 and over	31	3	96.8
Total		282	56.8

Genetic Aspects of Bovine Ocular Carcinoma

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The primary purpose of this investigation was to determine the role of genetic constitution in bovine ocular carcinoma ("cancer eye"), along with determination of some of the other etiological factors which account for variation in the manifestation of this malady. There is ample and definitive evidence of a genetic basis underlying many cancers in laboratory animals, but such evidence is not as prevalent in larger mammals.

The genetic approach to cancer eye is one phase of a co-operative study, which also includes the approaches of pathological development and anatomy (Russell, Wynne, and Loquvam, 1956; Wynne, 1958), tissue culture (Sykes, Dmochowski, Wynne, and Russell, 1958, 1959), and electron microscopy (Dmochowski, Sykes, Wynne, and Russell, 1958). The information thus obtained may be pertinent to cancer in man, as well as to its control in the bovine, and should contribute to general biological knowledge.

MATERIALS AND METHODS

The experimental animals were Hereford cattle. Since they generally constitute approximately 90 per cent of the beef cattle at the principal markets of the range areas, it seemed reasonable to initiate investigations of this eye malady in a prevalent breed. Though the frequency of the disease is thought by many to be higher in this breed than in the other breeds, factors other than breed differences per se

with ages from 5 to 15 years were included; that is, where relatively more animals were in the age range when most cases of cancer eye develop (Anderson, Chambers, and Lush, 1956). These heritabilities indicate that an earlier age at onset in the parent tends to be associated with an earlier age at onset in the daughter.

The heritability of 4 might be too high compared with one from a random sample of cattle of similar ages of this breed, if some factor(s) other than heredity were acting to increase the likeness between dam and daughter. However, this type of bias would be canceled in part, or perhaps wholly, by the effect of the genetic relationship which existed among the dams in the present sample. The effect of genetic relationship or consanguinity is to reduce the genetic variance among dams, thus reducing the part which heredity would play in causing phenotypic differences among them.

The evidence of a genetic basis in cancer eye susceptibility perhaps is seen more vividly in Table 2, which combines the two aforementioned groups of animals. Since the results from each group were similar, they were combined for brevity The daughters in each mating type were over four years of age (and therefore were in the age range when the preponderance of cancer eye lesions develops).

The proportions in the last column show that one affected parent increases the relative frequency of the disease by 12 5 per cent, and two affected parents increase it by 25 per cent, compared with the frequency of 11 per cent among daughters of two nonaffected parents. The average ages of the daughters in the mating types did not differ importantly; so this could not have accounted for the differences in frequency. The differences are larger than would be ex-

TABLE 2 Percentage Frequency of Cancer Eye in Daughters* of Affected and Nonaffected Parents

Condition of parents Sire × Dam	Number of matings	Percentage frequency and standard error
Normal × Normal	404	11 ± 1 5
Cancer × Normal	83	25 ± 4 8
Normal × Cancer	85	22 ± 4 5
Cancer × Cancer	14	36 ± 12 8
Total	586	17 ± 1 5

* Daughters over four years of age

Records complete enough for genetic analysis on 943 animals are the primary basis of the present report, though additional records on 4,960 animals are referred to in the discussion of age at onset.

Results and Discussion

Cancer eye is similar to cancers in other animals in being a disease of the aged. This is shown in Table 1 where the frequency of cancer eye per 1,000 female survivors to each age tends to increase from 0.8 for three-year-old animals to 96.8 for those over 14 years of age. The variability in frequency after 10 years of age probably reflects the small numbers of affected animals in these age classes.

Clearly, age is important and should be considered when interpreting reasons for the presence or absence of cancer eye. Age has been shown to be a measure of variability in susceptibility to certain tumors in mice (Heston, 1940). Differences in age at onset of cancer eye also appear to be determined in part by genetic differences. This was shown by an analysis wherein differences in susceptibility among genetically related animals were determined by differences in age at onset (Blackwell, Anderson, and Knox, 1956). The basic assumptions were: 1) Susceptibility is a continuous variable, in that animals developing lesions early in life are probably more susceptible than those developing lesions at older ages; and 2) among unaffected animals, the older ones are assumed to be more resistant since some among the younger ones probably would have developed lesions if they had been observed to older ages. Since the statistical distribution of susceptibility was assumed to be "normal," the derivation of the susceptibility score was based on the normal curve (Pearson, 1924). The arbitrary scores thus obtained, which for affected animals ranged from 25 to 3, and from 2 to 0 for unaffected animals, are by no means exact measures of susceptibility since they may attach too much (or too little) emphasis to the ages at which cancer developed. However, the susceptibility scores appear to be roughly proportional to the economic importance of the age at which this cancer develops.

The heritability of susceptibility; that is, the portion of all individual differences in the age score for susceptibility that is caused by genetic differences, was estimated by a regression of daughter's score on dam's score, involving 384 comparisons. A heritability of .29 was found. A similar age score, but derived from and applied to an independent group of 183 animals of similar ages (3 to 15 years), resulted in another estimate of .27, showing close agreement with the previous one. The estimate of .27 increased to .4 when only animals

with ages from 5 to 15 years were included; that is, where relatively more animals were in the age range when most cases of cancer eye develop (Anderson, Chambers, and Lush, 1956). These heritabilities indicate that an earlier age at onset in the parent tends to be associated with an earlier age at onset in the daughter.

The heritability of .4 might be too high compared with one from a random sample of cattle of similar ages of this breed, if some factor(s) other than heredity were acting to increase the likeness between dam and daughter. However, this type of bias would be canceled in part, or perhaps wholly, by the effect of the genetic relationship which existed among the dams in the present sample. The effect of genetic relationship or consanguinity is to reduce the genetic variance among dams, thus reducing the part which heredity would play in causing phenotypic differences among them.

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pected by chance, the respective probabilities being less than .01 for the combined reciprocal matings, and approximately .05 for the Cancer × Cancer type. Autosomal inheritance is indicated by the small difference in the proportions for the reciprocal matings. This small difference also indicates the absence of any pronounced maternal influence on susceptibility. The heritability of susceptibility is .25 when estimated by the regression of daughter on dam or on sire. This is obtained by doubling the difference of .125. The difference of .25 between the two extreme mating types yields a direct estimate of heritability from a regression of daughter on midparent. These estimates are lower than those for the measures of susceptibility, probably because age at onset is neglected here but was considered in the score.

The brownish-red pigment in the skin around the eyes of animals in this particular breed has a definite inhibitory effect on lesions. Lesions were never observed to develop in any pigmented area of the lids regardless of the shade of that pigment; i.e., light red, dark red, or brown. Lesions were observed, however, in nonpigmented areas of partially pigmented lids, but not as frequently as on lids with less pigment. The inhibitory effect of pigment seems to be a direct one confined only to lid lesions, and not extending beyond the pigmented areas themselves (Anderson, in preparation).

If the lesions represented in Table 2 had developed mostly on the lids (in the disposal data from one of the herds we have no way of knowing whether the lesions originated on the lids or on the eyeball) then the genetic basis being detected might be primarily that of pigment itself. In view of this, it was considered desirable to test whether a genetic basis existed for susceptibility, independent of these effects of pigment.

To this end, a better measure for detecting individual differences in susceptibility was developed. Since animals were observed for a 30-month period, beginning when they were six years of age, information was available showing the age at onset of an animal's first lesion, the number of sites on the eye affected by lesions, and the number of lesions which regressed. These three factors have been shown to measure adequately the variability in susceptibility to certain tumors among groups of mice or fowl of different genotypes (Heston, 1940; Greenwood, Blyth, and Carr, 1948), and were assumed to be similarly involved in the bovine. A young age at onset, and a high number of affected sites and a small number of regressions were considered to indicate a high degree of susceptibility, or a high

TABLE 3. Components of Variance for Circumocular Pigmentation and Lid, Eyeball, and Total Susceptibility for 105 Animals, Eight and One-Half Years Old

Source of variation	Degrees of freedom*	Expected mean squares	Components of variance, σw^2 σb^2			
			Lid susceptibility	Eyeball susceptibility	Total susceptibility	Pigmentation
Total	208					
Between pairs of eyes (b)	104	$\sigma w^2 + 2\sigma b^2$	4 929**	2 970**	8 862**	748 453**
Within pairs of eyes (w)	104	σw^2	2 519	4 938	10 404	577 077
	Intraclass correlations		66	38	.46	.56
	95% confidence intervals		.50–82	22–.54	.30–62	.40–.72

* One animal with one eye, thus 105 animals but 209 eyes
** $P < 01$

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* One animal with one eye, thus 105 animals but 209 eyes

** P < 01

score. Resistance was denoted by a low score. Normal animals thus received a score of 0, but only 8 of the 105 animals remained clinically normal throughout the 30-month observational period. The score for susceptibility was determined by simply adding the figures for age and sites and subtracting the number of regressions for each eye (Anderson, in preparation). The score also was computed individually for lid susceptibility and for eyeball susceptibility, because the latter is not influenced by lid pigment. This permitted evaluating genetic variation in susceptibility independently of the effects of pigment. Total susceptibility per animal, which is the sum of the lid and eyeball scores, was distributed nearly normally with a range in value from 0 to 31 and an average of 11.

The variation in this measure of susceptibility between and within pairs of eyes was analyzed. The variation within pairs of eyes is presumably wholly nongenetic, consisting of developmental errors during the embryological period, nonlinearity between the score of susceptibility and the primary gene effects, and environmental influences which affected one eye but not the other. The variation between pairs of eyes contains all the genotypic variance and all the variance caused by environment that is common to both eyes on the same animal. Shown in Table 3 are the components of variance between and within pairs of eyes for pigmentation and for susceptibility. It also shows the resulting maximal estimates of heritability and their approximate 95 per cent confidence intervals. These heritability estimates may be compared with conventional heritabilities, which contain little except additively genetic differences, to throw light on the nature of the variation in pigmentation and in susceptibility.

All the heritabilities are significantly different from zero. The estimate of .56 for circumocular pigmentation is slightly larger than a conventional heritability of .45 reported in a study including the present animals along with others (Anderson, Chambers, and Lush, 1957). The relatively small difference of .11 (.56 minus .45) could mean that interactions between allelic and nonallelic genes (dominance and epistasis) and environmental factors common to both eyes account for a small portion of the variation in amount of circumocular pigment; whereas additively genetic differences account for about .45. The remainder of .44, which is the variation between eyes on the same animal, is all due to nongenetic factors.

The estimates of .46 for total susceptibility and .38 for eyeball susceptibility compare favorably with the aforementioned conven-

tional heritability of .4 for total susceptibility (Anderson, Chambers, and Lush, 1956). Allelic and nonallelic interactions seemingly have little or no influence on susceptibility as it is being measured here. That the estimate for total susceptibility is only slightly larger than the estimate of .38 for eyeball susceptibility, which is independent of the effects of pigment, is probably due to the fact that lid lesions constitute a relatively small fraction (one-third) of all eye lesions in this particular sample of animals. The estimate of .66 for lid susceptibility is probably high because it includes the effects of pigment. A path-coefficient analysis (Wright, 1934) indicated that the correlation between lid susceptibility in the right and left eyes, after subtracting the influence of pigmentation being similar in the two eyes, would be approximately .50. There is evidence, therefore, of a genetic basis in susceptibility independent of the effects of pigment.

The tendency for cancer eye lesions to develop bilaterally also is shown in a summary of 1,190 pairs of eyes in animals that were at least five years of age. Among these animals, 229 had bilateral lesions, 150 had lesions only in the right eye, 161 had lesions only in the left, and 650 were normal in both eyes. If the lesions in the right and left eyes were independent only 124 animals were expected to have bilateral lesions. The probability is much less than .001 that the excess of bilateral involvements and the corresponding deficits of unilateral involvements could have occurred purely by chance if lesions in one eye were in fact independent of those in the other eye of the same animal.

These results indicate that many factors (over and above the influence of pigment) affect the susceptibilities of both eyes alike. The development of lesions bilaterally constitutes strong evidence for a genetic basis, but it also could be regarded as evidence for other etiological factors which tend to affect both eyes if they affect either. The similarity of heritabilities from data on bilaterality compared with heritabilities which contain little except the additively genetic variance, suggests that genetic factors account for most of these correlated effects to the extent that about 40 per cent of the phenotypic variability in susceptibility can be ascribed to additively genetic differences. Evidence of a genetic basis has practical applicability toward controlling this disease by selective breeding programs

It will be recalled that pigment has a direct inhibitory effect on lid lesions in that lesions do not develop in pigmented areas of the lids, though they do develop in nonpigmented areas of partially pigmented lids. Also, pigment has no inhibitory effect on any other type of

lesion in the same or opposite eye. These findings suggest that the gene loci for susceptibility are different from those for pigment, although loci for pigment appear operationally pleiotropic to lid susceptibility by their intermediate effect on pigment. If pleiotropy had been an important factor in these comparisons, then pigment would have been expected to manifest more generalized inhibitory effects than were actually observed.

The findings in general are somewhat similar to those reported for Negroids where the relative lack of skin cancer is explained by the degree of pigmentation of the skin (Steiner, 1954). Pigmentation is the factor reported to be inherited, and not resistance of the cells to neoplastic transformation. Albino Negroids are reported as being susceptible to actinic cancer.

The present findings indicate that susceptibility of the cells of the eye to neoplastic transformation and the potentiality for pigment formation both can be inherited. However, susceptibility might never become manifest in some individuals because pigment could shield the whole individual or merely some area of the body from a carcinogen (sunlight?), thereby preventing or at least hindering a threshold from being reached above which cancer would ensue. The threshold for susceptibility is assumed to depend upon a combination of genetic and environmental factors such that if the genotype places the individual near the threshold, the probability is high that environmental factors would push it over the threshold. If the individual is not highly susceptible genetically, the probability is small that environmental factors would push it above the threshold early in life, but might increase as the individual becomes older.

Summary

Bovine ocular carcinoma was described as a disease of the aged, since it showed marked increases in relative frequency with age in a sample of 4,960 animals. Genetic differences appeared to account for about 40 per cent of the phenotypic variability in susceptibility when susceptibility was measured by age at onset of lesions alone; or by age at onset, number of affected sites, and number of spontaneous regressions. The gene loci for susceptibility and loci for pigment did not appear to be the same. The findings indicate that both susceptibility of the cells of the eyes to neoplastic transformation and pigment potential are heritable, though the presence of pigment may hinder or prevent the manifestation of susceptibility.

ACKNOWLEDGMENTS

The investigation was supported in part by Grant G-1 from the American Cancer Society, Grants C-1751 and C-2890 from the National Cancer Institute of the National Institutes of Health, Public Health Service This is publication Number 7 of the Cancer Eye Study Section The author wishes to express his appreciation to Drs D. Chambers and J Lush for their valuable suggestions during the various studies.

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HEREDITY AND HUMAN CANCER

Genetics of Man:
Some of the Developments of the Last Decade

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Twenty years or so ago, one would have begun a report on the "Genetics of Man" by attempting to justify man as an object of genetic inquiry. This, fortunately, is no longer necessary. Recent events, some of which will be cited, illustrate contributions to genetic thought which can be expected from the study of man. I might also add that one rarely feels called upon to justify an area of research which is attracting as much attention as human genetics currently attracts. There presently prevails a far greater acceptance of human genetics as a scientific discipline than has prevailed at any time in the past. To a large extent the reasons for this acceptance are to be found in (1) the increasing preoccupation of medicine with chronic and degenerative diseases where, a priori, causation would appear in large measure dependent upon one's genetic background, and (2) the emergence of techniques, largely biochemical, whereby it is possible to approach more nearly the level of primary gene action than has been possible in the past.

We shall consider here, briefly, certain advances in our understanding of: (1) The genetics of the red blood cell, (2) the methodology of human genetics, and (3) the genetic component in cancers of man. The first of these is included because it illustrates the level of scientific sophistication which can be reached in only a few areas of human genetics at present, although we hope that it will typify more and more areas of genetic investigation in the future.

Genetics of the Red Blood Cell

The past decade has seen rather phenomenal progress in our understanding of the antigenic makeup of the red blood cell, of its hemoglobin, and of some of the intracellular enzyme systems not immediately related to hemoglobin. Perhaps the most fascinating part of this advance has to do with the inherited abnormalities of hemoglobin. Events in this area have moved so rapidly that it is difficult to believe that less than 10 years have elapsed since the publication of the three papers which triggered this avalanche of activity. The papers in question are those of Beet (1949), Neel (1949), and Pauling, Itano, Singer, and Wells (1949). Almost simultaneously, Beet and Neel postulated that sickle-cell anemia was the consequence of homozygosity for a gene which in single dose gave rise to individuals whose red cells would sickle *in vitro*, but who otherwise were clinically normal. Literally within weeks, Pauling and his associates had clearly demonstrated, by the atypical behavior of the hemoglobin on electrophoresis, that the basic defect associated with the gene was at the molecular level. At the present time, in the neighborhood of two dozen inherited variants of normal hemoglobin are known.

The genes controlling the different hemoglobins differ widely in their frequencies, with those associated with hemoglobins S and C being particularly common in Negroes, the one associated with hemoglobin D being common in Asiatic Indians, and the one responsible for hemoglobin E being common in the Thai. From the classic genetic viewpoint, we have good evidence that *at least* two loci are involved in the production of these variants. Data from families in which more than one of the genes in question are segregating suggest that the genes responsible for hemoglobins S and C are, in all probability, allelic, whereas the one for hemoglobin G is nonallelic to S and C (see Ranney, Larson, and McCormack, 1953; and Schwartz, Spaet, Zuelzer, Neel, Robinson, and Kaufman, 1957).

One of the many intriguing developments in connection with the hemoglobins has been Ingram's (1957) report that hemoglobins S and C differ from the normal by a single amino acid in what he has designated as the No. 4 peptide. In the case of hemoglobin S, a valine is substituted for glutamic acid; and in hemoglobin C, a lysine replaces the same glutamic acid. One of the more pressing needs in connection with this finding is the demonstration that each of the peptides in hemoglobins S and C which has the same electrical charge and solubility in butanol-acetic acid as a peptide in normal hemo-

globin is, in fact, identical in amino acid composition and sequence with the corresponding peptide in hemoglobin A. Ingram's biochemical technique of analyzing the hemoglobins would not necessarily detect an amino acid substitution which did not lead to a difference in the net charge of the peptide. At the moment, the chemical difference jibes rather neatly with the segregation behavior of the genes responsible for hemoglobins S and C.

Progress in the more rigorous biochemical characterization of the hemoglobins will undoubtedly continue at a rapid rate. Foretokening this is the demonstration that hemoglobin D, initially described by Itano (1951) and found in a variety of areas in the world, may involve a number (three are now recognized) of different biochemical entities having in common an electrophoretic mobility similar to S hemoglobin, but differing from the latter in solubility (Benzer, Ingram, and Lehmann, 1958). The implications of this finding are many. It is certainly not inconceivable that further analysis of the hemoglobins may give rise to the human counterpart of the rII mutants in the bacterial virus T4 (Benzer, 1957).

To many, the insight into the dynamics of human population genetics afforded by the hemoglobinopathies is no less interesting than the information which we have gained from them on gene action. Allison (1954) was the first to champion vigorously the thesis that the high frequencies of the sickle-cell gene in Africa were attributable to a balanced polymorphic system wherein the heterozygote enjoyed a selective advantage as a consequence of increased resistance to malaria. The evidence advanced by Allison was circumstantial, in the main, being a correlation between the frequency of the sickle-cell gene and the endemicity or hyperendemicity of malaria. Efforts by Allison (1954), and by Beutler, Dern, and Flanagan (1955), to demonstrate directly that the heterozygote was more resistant to infection with *Plasmodium falciparum* led to somewhat conflicting results. Be this as it may, the intervening years have seen the accumulation of additional data in support of Allison's general thesis. Excellent reviews of this evidence and the problem of documenting the association of malaria with the sickle-cell gene are to be found in Neel (1956) and Livingstone (1958). At the present, there are few who do not believe that there is a causal relationship between the incidence of malaria and the frequency of the sickle-cell gene, and, perhaps, the gene responsible for hemoglobin C as well. The precise basis of the relative immunity which must be involved is not known, although there are a number of clues, among these being Raper's

(1956) data which suggest that sicklers do not suffer from cerebral malaria. There are other findings on the heterozygote, such as hyposthenuria, which have not been fitted into the puzzle as yet. With respect to hyposthenuria, there are reasons for believing that the lowered specific gravity of the urine of the heterozygote reflects a renal pathology (Rucknagel, personal communication). If this is so, we have the rather interesting situation where the heterozygote operates at an advantage in a malarious zone, but at a disadvantage in a nonmalarious area. Moreover, the advantage of the heterozygote in Africa, say, must be such as to replace not only genes lost through the death of persons with sickle-cell anemia but possibly also some fraction of heterozygotes whose fitness is below average because of renal disease.

A variety of hypotheses has been advanced regarding the mechanism of increased fitness of the heterozygote. Recently, for example, Livingstone (1957) has suggested that the increased fitness of the sickle-cell heterozygote may be mediated to a large degree through the carrier female. He believes that *P. falciparum* infections of the placenta may not develop to the same extent in sickling as opposed to nonsickling mothers, and that as a consequence the former would have a proportionately higher number of live-born children. Much of the available evidence is consistent with this hypothesis. Edington (1955), for example, finds that sickling mothers have a slightly lower stillbirth rate, and that their infants have a higher mean birth weight and a higher survival rate. There is as yet, however, no direct evidence that placental infections are either less common or less severe in sickling mothers.

Another lesion of the red blood cell which has been detected recently is the deficiency of glucose-6-phosphate dehydrogenase associated with individuals demonstrably sensitive to the antimalarial primaquine, as well as to a growing list of the other drugs (for a review of this work see Beutler, 1959). Work on this inherited abnormality is proceeding at such a feverish pace that any attempt to summarize progress will be dated almost as rapidly as one sets pen to paper. At the risk, however, of jousting with what might ultimately prove to be a straw man, I should like to call attention to just one aspect of this anomaly; namely, the marked differences observed in the two sexes. Two alternative explanations are available: (1) the trait may be sex-influenced, and (2) the trait may be sex-linked as suggested by Childs, Zinkham, Browne, Kimbro, and Torbert (1958). In possible favor of the former is the report that hysterectomy has

led, in one instance, to a switch from a normal to a sensitive level of G-6-PD as measured by the glutathione stability test. At the moment, the bulk of the data would appear to favor the sex-linked hypothesis, although even here there are a number of anomalous findings which usually are accounted for on the basis of the classification difficulties in the female. Be this as it may, if Child's hypothesis is correct, one's interest naturally turns to the problem of what maintains this gene in primitive populations. If we are confronted again with a balanced polymorphic system, it will be one unlike any previously seen in man. Interestingly, a balanced system involving a sex-linked gene can be maintained with or without an advantage to the heterozygous female. Specification of the selective advantage or disadvantage conferred upon each of the five possible genotypes presents a challenging problem. A complication in any development of the population implications of primaquine sensitivity is the report of Alving, Kellermeyer, Tarlov, Schrier, and Carson (1958) that there are no substantial differences between males and females to *in vivo* challenge with primaquine despite the apparent differences in the sexes in the levels of G-6-PD. Clearly, one can speculate at considerable lengths here, but in the absence of evidence that individuals with G-6-PD deficiency are at a disadvantage in primitive societies, and that the gene exists in appreciable frequencies in such societies, speculations may be noncontributive.

Our knowledge of the genetic control of variation in the red blood cell has been the subject of numerous reports. Obviously then, one cannot do justice to this area in a few moments. Its importance in the present context seems to me to be one of: (a) pointing a goal toward which we might strive in other areas of human genetics, and (b) demonstrating the necessity of the appropriate tool before one can make much progress in the understanding of a complex phenomenon. The genetics of the red cell would not have reached its present stage of development if morphological differences in the cells were the sole means of classification.

Advances in Methodology

In a consideration of the advances of the last decade, one can hardly overlook the strides which have been made in providing us with the analytical tools necessary for extracting the maximum information from the data which we collect. Here, however, it is more difficult to single out important contributions because frequently what at first glance appears to be relatively unimportant may, in the

hands of others, become the source of a major breakthrough. At the risk of being patently unfair to those who have worked largely on population theory—and there have been important developments here, for example, Kimura's work on stochastic processes and the distribution of gene frequencies under natural selection—I shall restrict my attention to two areas of more immediate applicability to human genetics.

In formal human genetics, one of the major methodological advances has been, in my estimation, the development of more potent *tools for the study of linkage. A decade ago we had two procedures* for linkage detection; namely, the paired-sib method of Penrose and the Finney modification of Fisher's u-statistics (this ignores the analysis of single, large pedigrees by backward odds). Both of these procedures employed samples of fixed size. Each had recommendations, but neither fully met the needs of the day. The first change to be ushered in was Bailey's (1951) generalized method of determining the appropriate multiple of the u-score to be used in linkage testing. While this did not alter basically the tests which were available, I believe that it is fair to credit Bailey with an important role in redirecting attention to linkage tests. In an excellent paper on the detection of linkage in man, C. A. B. Smith (1953) suggested that odds or likelihood ratio tests of significance offered advantages not to be found in tests based upon efficient scores. He and Haldane (Haldane and Smith, 1947) had previously applied this procedure to the estimation of the linkage between the genes responsible for hemophilia and color blindness. The next step was the presentation by Morton (1955, 1957), of a *sequential procedure of linkage detection* based upon the sequential probability ratio test developed largely by Abraham Wald. This procedure was appreciably simpler, more convenient in practice, and, in general, more efficient, than most of the previously used methods. Morton applied the method with success to the problem of the linkage between the Rh locus and elliptocytosis and to the matter of partial sex linkage (1957). At this point, it seemed as though further refinement of linkage tests might outstrip the quality of the information available for such tests. The next advance, however, was to come in quite a different direction, and again it was C. A. B Smith to whom we are indebted Smith (in press) has, in effect, challenged the whole orientation toward tests of significance in the matter of detecting linkage. He argues that in linkage procedures we are, or should be, interested not in a definite decision (to accept or reject the hypothesis of no linkage), "but rather in a measure of confi-

dence in or plausibility of a hypothesis." He then proceeds to show that with Bayes' Theorem and the initial distribution for recombination fractions given by Morton (1955) it is possible to present the results of linkage tests in probability form Smith's paper ultimately may have importance well beyond linkage tests in leading us to reinvestigate the objectives which we seek to attain with the application of statistical methods to genetic data.

The second area where strides have been made is in part an outgrowth of efforts to evaluate the risk accruing to mankind from increasing exposure to ionizing radiations. The value which one places on this risk will be a function of whether one views the bulk of selection as favoring homozygotes ("the classical hypothesis") or heterozygotes ("the balanced hypothesis"). Until recently, and as a matter of fact at the present, one could argue for either of these alternatives, so far as man is concerned, without fear of contradiction. Of late, however, there have been several indications that this state of affairs will not persist too much longer.

Crow (1958) has presented certain possibilities for measuring selection intensities in man. Of interest to us here is the genotypic selection intensity that Crow has termed "the genetic load of a population." He sees this load as subdivisible into a number of components, of which mutation and segregation are two of the more important. With the aid of a rather simple model, he proposes means whereby one can measure the mutational and segregational loads One of these methods, and the one for which we presently have a sizable body of data, is the differential response of these two components to inbreeding. Briefly, the method consists of contrasting the mutational (or segregational) load in a randomly mating population with that in a homozygous population as inferred from the results obtained in studying the children of consanguineous marriages For the mutational load, it can be shown easily that the ratio of the effect in an inbred population to that in a randomly mating population is $1/2h$ where h is the average dominance of nominally recessive genes; whereas the corresponding ratio for the segregational load is 2 for a two-allele locus or k for a k-allele locus. The first application of this notion to human data was by Morton, Crow, and Muller (1956). Subsequent efforts along these lines (Schull, 1958; Slatis, Reis, and Hoene, 1958) have led to surprisingly similar results. In the data currently available, the ratio of the inbred to random-mating effect, as measured in terms of mortality, is in the neighborhood of 15 to 25. As Crow has pointed out, on the "balanced hypothesis" this would

lead to postulating 15 to 25 alleles at a locus, which does not seem reasonable; whereas this value would be compatible with an average dominance of 0.020 to 0.033 per locus, a value consistent with Drosophila data. Crow quite naturally arrives at the conclusion that "ordinary" genes are relatively more important in selection than polymorphic balance. Now, one can attack this argument at at least two levels. First, the very simplicity of the model can be attacked on the grounds that it is an oversimplification of the true situation; second, one can attack certain particulars of the argument, and perhaps the weakest of the latter links is our lack of knowledge of the average dominance of nominally recessive genes in man. Crude estimates of the value of h in man should be obtainable without too much difficulty; precise estimates would be more difficult at our present stage of development.

Finally, before leaving this section, permit me to point to one area in methodology where we have not made much progress. In the analysis of much of the data on man, we continue to be plagued by the rather peculiar samples, if they can be so designated, which come to our attention. Rarely does one see employed a sampling system which would meet with the approval of the specialist in sampling theory. We continue to be guided largely by pragmatic considerations and to take our data from whatever source they may be conveniently gleaned. The net result of this is that the inferences which we wish to draw regarding the population are based not upon probability samples but upon "chunks" of data. The statistical theory of inference covers the former, but it certainly would not cover the latter unless the "chunk" amounted to virtually the entire population. Progress in this direction is possibly as much a matter of education as the development of new theory, since a number of potent statistical tools, e.g., discriminant functions, either parametric or nonparametric, have seldom been used in the analysis of data in human genetics.

Advances in Our Understanding of the Genetic Component in Cancers of Man

My justification for introducing this subject is an attempt to provoke a reappraisal of the techniques which we have been using, and particularly those employed in studying the cancers of more common occurrence.

At the outset, I should like to draw a rather sharp line between those studies directed toward the rare malignant tumors of man, such as retinoblastoma or multiple polyposis of the colon, and those

directed toward cancers such as of the breast, stomach, cervix, or rectum, which lead to the death of approximately one out of eight persons in the United States. With respect to the former class, studies designed along the classic family approach have been relatively profitable. Thus, in multiple polyposis of the colon we have been able to enhance our knowledge of (1) the fitness, as measured in Darwinian terms, of individuals with this disease, (2) the distribution of age at onset, and, to a lesser extent, (3) intrafamily correlation in age at onset. Similarly in retinoblastoma we have begun to partition the inheritable from the noninheritable sporadic occurrences of this disease. There remain, of course, many unsolved problems. In retinoblastoma, for example, we do not know what proportion of the noninheritable sporadic occurrences are instances of phenocopying and what proportion are instances of somatic mutation.

With these rare cancers, we can come to grips with not only a variety of genetic problems, but we can construct genetic models which are amenable to testing. It seems to me as though we really have not begun to exploit fully the information on the cancerous process to be gained from these rare tumors. We have, for example, no idea as to why sarcomatous metaplasia occurs in one individual with neurofibromatosis and not in another Nor, for that matter, do we know whether sarcoma is randomly distributed among individuals with neurofibromatosis or whether it tends to cluster in families. The same could be said for osteosarcoma arising in individuals with multiple exostoses. What relationship does the leukemoid reaction that one sees in certain individuals with infectious disease bear to leukemia, and is this leukemoid reaction characteristic of other members of the family of the individual in question?

Answers to these questions and others which could be formulated readily would certainly have relevance to the conflicting theories of the origin of cancer. As a case in point, consider Professor Darlington's theory of the importance of plasmagenes in cancerogenesis. Now I find this theory a rather intriguing explanation for the rampant cell growth associated with cancer, but I'm not willing to accept as yet the generalization that nuclear genes play a small role. In multiple polyposis of the colon, for example, the probability that one of Darlington's plasmagene mutations will occur in one or more of the cells of the bowel must be almost 1 The fact that the occurrence of a plasmagene mutation can be so influenced by a nuclear gene hardly implies, to me, a small role for the nuclear gene. Professor Darlington pointed out the dangers of generalization when one deals with

what would appear to be as complex a biological phenomenon as cancer. To this I should like to add that extrapolations from *in vitro* cells or highly inbred strains of flies or mice to what happens in man are no less dangerous. Sooner or later our theories must be put to test on man. What better area to be looking for answers than in those individuals who with near certainty will develop cancer. Surely the answers will not come until we can study the sequence of events which precedes that pathologic state which we call cancer, and it seems to me highly unlikely that this sequence of events will emerge from a retrospective study.

As one scans the literature on genetic factors in the more common cancers, one cannot help but be impressed by the frequently acrimonious and, to me, generally puerile polemic on "controls." This is the favored device of the statistician for obfuscating an issue. One is impressed by the surprising uniformity of findings despite the plethora of different controls in the various studies. This leads one to speculate on the value of the present approach Thus, in a recent study (Woolf, 1955) of the genetic aspects of carcinoma of the breast and stomach, for example, the objectives of the study were said to be "(1) to determine whether familial tendencies could be shown for the two types of neoplasia, (2) to determine the magnitude of these tendencies, if present, and (3) to determine whether the familial tendencies, if present, are limited to specific organs or are of a more general nature that predispose to cancer irrespective of location." A good many man-hours later, the author is able to conclude that (1) relatives of the stomach cancer propositi exhibit a significantly higher frequency of stomach cancer deaths than expected in the general population (two-to threefold increase); (2) female relatives of breast cancer propositae exhibited a significantly higher proportion of deaths from breast cancer than expected in the general population (two- to threefold increase); and (3) in both breast and stomach carcinoma the inherited tendency was organ-specific. Similar findings have been reported for cancer of the breast by Jacobsen (1947), Penrose, MacKenzie, and Karn (1948), Macklin (see pp. 408 to 425, this volume), Oliver (see pp. 426 to 438, this volume), and Anderson (1954). All of these workers find approximately a two- to threefold increase in breast cancer among the female relatives of breast cancer index cases. Studies of cancer of the corpus of the uterus (Brøbeck, 1949), and of the cervix and corpus combined (Murphy, 1952) result in findings comparable to those for cancer of the breast. And finally, Videback's study (1947) of leukemia in Denmark follows

the same general pattern found in cancer of the breast, uterus, and stomach.

Now what do these results tell us about the role of genes in the etiology of cancer? To be argumentative, I will take the position that they tell us virtually nothing. It seems to me that we might have anticipated the general level of effect which is detected. Certainly clinical impressions alone, subjective though they may be, would lead one to expect a relatively small increase in risk to the relatives of index cases. That there would be an increase and that it would be small follows from the correlation among relatives not only of genotypes but of environments as well, and a large increase could hardly escape clinical recognition. We are, it seems to me, destined to find a small effect, and this is what we find. That this effect cannot be interpreted in meaningful genetic terms may stem from the heterogeneous nature of the end point which we measure. Personally, I fail to see how the present approach can ever materially advance our understanding of the origins of the more common cancers.

A more profitable approach would seem to be a concentration on known high risk families, and a concerted effort to exploit the untapped information in the rare cancers. I certainly do not envisage a study oriented along the usual lines, retrospective and with emphasis on simple pathologic classification. Rather, an effort toward eliciting biochemical or physiologic differences among individuals within the aforementioned families might provide the "handle" whereby one gains entry into the problem. Finally, let me clearly state that I do not mean to imply that studies such as the ones we have just mentioned are totally without value. These studies have provided important demographic information, and they have served to reinforce certain impressions and areas of our knowledge. I am, however, opposed to the needless replication of these studies which has occurred in the past. I can see no useful purpose served by an effort to add "decimal points" to the risk of occurrence among relatives of probands with breast, stomach, etc., cancer.

In closing, permit me to say that one of the advantages of being the proverbial "fly in the ointment" is that it absolves one of constructive recommendations. However, to the extent that a re-examination of methods may in itself be constructive, the role of the fly is one which I find not entirely unrewarding.

what would appear to be as complex a biological phenomenon as cancer. To this I should like to add that extrapolations from *in vitro* cells or highly inbred strains of flies or mice to what happens in man are no less dangerous. Sooner or later our theories must be put to test on man. What better area to be looking for answers than in those individuals who with near certainty will develop cancer. Surely the answers will not come until we can study the sequence of events which precedes that pathologic state which we call cancer, and it seems to me highly unlikely that this sequence of events will emerge from a retrospective study.

As one scans the literature on genetic factors in the more common cancers, one cannot help but be impressed by the frequently acrimonious and, to me, generally puerile polemic on "controls." This is the favored device of the statistician for obfuscating an issue. One is impressed by the surprising uniformity of findings despite the plethora of different controls in the various studies. This leads one to speculate on the value of the present approach. Thus, in a recent study (Woolf, 1955) of the genetic aspects of carcinoma of the breast and stomach, for example, the objectives of the study were said to be "(1) to determine whether familial tendencies could be shown for the two types of neoplasia, (2) to determine the magnitude of these tendencies, if present, and (3) to determine whether the familial tendencies, if present, are limited to specific organs or are of a more general nature that predispose to cancer irrespective of location." A good many manhours later, the author is able to conclude that (1) relatives of the stomach cancer propositi exhibit a significantly higher frequency of stomach cancer deaths than expected in the general population (two- to threefold increase); (2) female relatives of breast cancer propositae exhibited a significantly higher proportion of deaths from breast cancer than expected in the general population (two- to threefold increase); and (3) in both breast and stomach carcinoma the inherited tendency was organ-specific. Similar findings have been reported for cancer of the breast by Jacobsen (1947), Penrose, Mackenzie, and Karn (1948), Macklin (see pp. 408 to 425, this volume), Oliver (see pp. 426 to 438, this volume), and Anderson (1954). All of these workers find approximately a two- to threefold increase in breast cancer among the female relatives of breast cancer index cases. Studies of cancer of the corpus of the uterus (Brøbeck, 1949), and of the cervix and corpus combined (Murphy, 1952) result in findings comparable to those for cancer of the breast. And finally, Videback's study (1947) of leukemia in Denmark follows

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Methods of Study in Human Genetics

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Human genetics is derived from four sources. The speculations and laws of experimental genetics give stimulus and rigor. Medicine, biochemistry, and immunology define the characters with which the human geneticist works. Tissue culture and transplantation hold forth the prospect of an experimental genetics of human somatic cells. Finally, methods of probability and statistics have led to the development of a nonexperimental genetics as precise within its limits as any experimental science.

This paper is concerned largely with the elements that distinguish human from basic genetics, and, therefore, with the methods of probability and statistics Starting with the pioneer contributions of German and English workers, and aided by modern developments in statistics and computing machines, methods adapted to the experimental restrictions of human genetics have been devised which are capable of resolving even very similar phenomena, like partial sex linkage and sex-biased manifestation; or incomplete penetrance, mutations, and phenocopies Distinctions can be made in man between mutations in egg and sperm, between time-dependent and division-dependent mutation rates, and between slight heterozygote advantage or disadvantage, with a precision which so far has not been approached in any other vertebrate, nor excelled in any laboratory animal The potential limits of nonexperimental genetics still are not

* Genetics paper No 718.

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defined clearly, but enough progress has been made to answer affirmatively the question of whether, even with restricted experimentation, a powerful and rigorous methodology can be developed for human genetics.

Linkage

Progress in methodology is typified by techniques for studying human linkage. The principles of linkage were well known from experimental organisms, when Snyder in 1926 suggested that linkage could be recognized in human pedigrees covering at least three generations. Bernstein in 1931 showed that linkage could be detected even if only two generations were given. His method assigned to each sibship a score, the expected value of which was determined by the recombination frequency. The linkage test consisted in comparison of the deviation of the total score with its standard error, both calculated on the hypothesis of no linkage. It was assumed that the amount of data was large enough so that the distribution of the total score in the absence of linkage would be approximately normal. This large-sample theory was refined by Hogben, Haldane, Fisher, Finney, Bailey, and Smith, until it seemed that every bit of information could be wrung from human pedigrees. Penrose extended these applications to sibs from unspecified parents, which increased the generality but decreased the efficiency and reliability of the linkage test.

Despite these developments, no linkages were discovered in man

TABLE 1 The Principle of Linkage Detection When the Phase Is Not Known Gametes Produced by a Parent Heterozygous for Two Linked Gene Pairs, G,g and T,t. The Frequency of Crossing-over Is θ

Parental Phase	Relative Frequency	Gametes GT	Gt	gT	gt	Total
GT/gt	$\frac{1}{2}$	$\frac{1}{2}(1-\theta)$	$\frac{1}{2}\theta$	$\frac{1}{2}\theta$	$\frac{1}{2}(1-\theta)$	1
Gt/gT	$\frac{1}{2}$	$\frac{1}{2}\theta$	$\frac{1}{2}(1-\theta)$	$\frac{1}{2}(1-\theta)$	$\frac{1}{2}\theta$	1
Average for both phases		$\frac{1}{4}$	$\frac{1}{4}$	$\frac{1}{4}$	$\frac{1}{4}$	1
Observed number in family		a	b	c	d	s

Probability ratio $= 2^{s-1}\,[\theta^{b+c}(1-\theta)^{a+d} + \theta^{a+d}(1-\theta)^{b+c}]$

for 20 years after the possibility of linkage detection was first explored. In fact, a number of claims were put forward which subsequently proved false. As the available methods were being used, they lacked precision and specificity. There were several reasons for this. Even in a large collection of families, usually only a few give appreciable information about linkage of any particular pair of loci, and in samples of this size the assumption of normality may be far from true. Since man has 22 pairs of autosomes, two loci taken at random are unlikely to be linked, and therefore the usual significance levels provide inadequate protection against false claims of linkage. For example, most assertions of linkage based on the conventional .05 significance level are expected to be incorrect, even in samples large enough for the calculated significance level to be valid, but significance at the .001 level provides strong evidence of linkage by a reliable test. The large-sample theory is not fully efficient for close linkage and does not extract full information from pedigrees. It has other disadvantages, including the complex calculations to which it leads.

Largely to avoid the assumption of normality and simplify computations, Haldane and Smith (1947) introduced a method based on the ratio of the probabilities of the observations with and *without* linkage By it they demonstrated close linkage of the loci for hemophilia and color blindness, both of which were known to be on the X chromosome Previously, Wald had extended probability ratio theory to sequential analysis and demonstrated the optimum character of this test. Since linkage data in man are perforce collected sequentially, a few families at a time, Morton (1955 *et seq.*) applied sequential analysis to linkage detection in man and showed that it is the most simple, exact, and efficient of available methods, requiring less than one-third as many observations for a given risk of error as the best alternative test. Tables of additive scores reduce computation to a minimum. The tables give efficient estimates and homogeneity tests, and provide accurate results in cases where other methods lead to false claims of linkage.

In recent years three cases of close autosomal linkage have been established in man, between the nail-patella syndrome and the ABO blood groups, elliptocytosis and Rh, and the Lutheran antigen and ABO secretor. Recombination takes place in both sexes with approximately equal frequency. The first of these linkages is homogeneous, indicating that the nail-patella syndrome is determined by the same locus in different pedigrees. Recombination between elliptocytosis

and the Rh locus is heterogeneous among pedigrees, and analysis demonstrates that hereditary elliptocytosis may be determined by either of two dominant factors, at present distinguishable only by the fact that one of them is closely linked to the Rh locus. This is the first instance in man of resolution of genetic entities by linkage to another marker, a technique that has great promise for the incisive analysis of rare factors.

Until a few years ago it was believed, on the basis of both genetic and cytological evidence, that some loci in man were on the homologous part of the X and Y chromosomes, and showed a characteristic mode of inheritance. More and more rare genetic diseases, like xeroderma pigmentosum, were claimed to show this "partial sex linkage." Meanwhile, cytological evidence against this phenomenon became impressive, and it was suggested that the genetic evidence is not specific for partial sex linkage, but could be explained equally well by sex-biased manifestation. That these diseases are not, in fact, partially sex-linked, was shown by Morton (1957) by a method which depends on the mode of transmission for two generations and reduces formally to the probability ratio scores for ordinary linkage.

Mutation

Human mutations can be studied in two classical ways. The "direct" method depends on complete ascertainment of new occurrences of a fully penetrant dominant gene among the offspring of normal parents. The "indirect" method uses the equilibrium relation between incidence, fitness, and the mutation rate, and is applicable to sex-linked and dominant genes and, assuming a single locus with no heterozygote expression, to autosomal recessive genes as well. Recently, a third method has been developed, which might be called "semidirect," in that it depends on estimation from segregating families of the frequency of sporadic cases among all cases in the population, and of the proportion of all sporadic cases that are mutants (Morton, 1958). Differing from the direct method in that sporadics are estimated, not counted, and from the indirect method in that no equilibrium condition is assumed, it is applicable to dominant genes with incomplete penetrance, sex-linked loci, and mixtures of genetic cases and somatic mutations or phenocopies, with no restriction about completeness of ascertainment. It is the method of choice for such conditions as retinoblastoma, spherocytosis, and dominant and sporadic deaf-mutism.

The direct, indirect, and semidirect methods all estimate mutation

rates toward a particular phenotype, but provide no internal test of whether this phenotype is determined by more than one locus This limitation is particularly troublesome for rare recessive genes, but fortunately there is an alternative approach based on inbreeding, the method of "detrimental equivalents" (Chung, Robison, and Morton, in press). Consanguineous marriages produce children with a greater risk of homozygosis for deleterious genes, the mean number of homozygous loci increasing in proportion to the inbreeding coefficient. From the population incidence at birth, the mean inbreeding coefficient of probands, and the segregation analysis, it is possible to obtain estimates of two quantities. the weighted sum of the recessive gene frequencies, and the weighted sum of squares of the gene frequencies, the weights being the penetrance in homozygotes and the summation being over all loci that can produce the phenotype in question. If the segregation analysis indicates that penetrance is complete in homozygotes, the first quantity reduces to Σq and the second to Σq^2, where q is a recessive gene frequency. If the recessive mutants are allelic, the expected value of $(\Sigma q)^2/\Sigma q^2$ equals one. In general, $(\Sigma q)^2/\Sigma q^2$ is an unbiased estimate of n, the number of loci that can produce this phenotype, if the recessive gene frequencies are equal at all relevant loci, otherwise, this ratio provides a minimum estimate. Then $\Sigma q/n$ estimates the mean gene frequency per locus, and from this the mutation rate per locus and per genome can be obtained by the indirect method, without assuming that the mutants are allelic

The theory of "detrimental equivalents" is an extension of the theory of "lethal equivalents" (Morton, Crow, and Muller, 1956), which estimates the total subvital mutation rate from mortality among children of consanguineous marriages This turns out to be 06–15 per gamete per generation, a value which is remarkably similar to independent calculations from mouse and Drosophila experiments It seems unlikely that this estimate is inflated appreciably by the "segregation load" of genes that are deleterious in homozygotes but advantageous in heterozygotes, because such factors could give the observed results only if the number of mutually heterotic alleles at each locus were very large. The evidence against this hypothesis is decisive for detrimental equivalents, since several hundred mutually heterotic alleles, each vanishingly rare, would have to be assumed at each locus to maintain the hypothesis of appreciable heterozygote advantage. The consanguinity data are inconsistent with such small gene frequencies and with so many genes The selection pressures that must be postulated are infinite-

and the Rh locus is heterogeneous among pedigrees, and analysis demonstrates that hereditary elliptocytosis may be determined by either of two dominant factors, at present distinguishable only by the fact that one of them is closely linked to the Rh locus. This is the first instance in man of resolution of genetic entities by linkage to another marker, a technique that has great promise for the incisive analysis of rare factors.

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The direct, indirect, and semidirect methods all estimate mutation

dwarfing, and deafness, while heterozygous carriers of the gene for phenylketonuria differ from normal homozygotes in serum phenylalanine and tyrosine levels before and during a phenylalanine tolerance test.

The most accurate discrimination of phenotypes requires a weighted combination of these different criteria. A satisfactory system of weights which maximizes the intraclass correlation may be determined from the theory of discriminant functions, by which Chung and Morton (1958) have been able to separate genetic classes of muscular dystrophy on clinical and biochemical evidence, to prove the existence of two clinically indistinguishable genetic types of limb-girdle muscular dystrophy, and to demonstrate that dominant, recessive, sex-linked, and sporadic types cannot be subdivided into more homogeneous genetic categories from present data. Discriminant functions should become routine methods for many genetic problems, including carrier detection and resolution of genetic entities.

Segregation Analysis

The basic data of genetics consist of the frequencies with which a trait appears in different types of matings. In human genetics, some trait bearers are selected through hospital records or other direct means of ascertainment. These cases found by direct methods are called *probands*, in contrast to secondary cases ascertained through investigation of the families of probands If the children of probands or their affected relatives are sampled without regard to their own phenotypes, we speak of *complete selection* of *these sibships*. Selection is said to be *incomplete* if only sibships with an affected member are sampled. This type of sampling generates a continuum of simple possibilities, ranging from selection so incomplete that two probands never occur in the same sibship (*single selection*), to selection so thorough that sibships with many cases are no more likely to be sampled than sibships with only one affected case (*truncate selection*). These possibilities may be described in terms of the *probability of ascertainment* (π), which equals 1 for truncate selection and approaches 0 for single selection, the intervening range constituting *multiple selection* Since π represents the probability that an affected person in the population be a proband, estimation of π is necessary not only for genetic analysis but also for determining the incidence of cases in the population.

In simple situations, for any phenotypic class of mating (such as normal × normal, affected × affected, or normal × affected), we

simal by comparison with observed mutation rates. It is incredible that so many rare alleles could be maintained by weak selection in a finite population. This is almost the only instance in any organism where it can be demonstrated that the frequency of a rare recessive is determined by mutation pressure rather than by heterozygote advantage.

Penrose (1955) has found that paternal age at the time of conception of dominant mutants is elevated, with no independent effect of mother's age. Morton and Chung (1958) were unable to demonstrate any increase of maternal age at the time of conception of isolated cases of sex-linked muscular dystrophy, many of which were new mutants. These observations indicate that mutations in man are less intimately related to gonial age than to the number of previous cell divisions, which increase with age for spermatogonia but not oögonia.

Recessive sex-linked mutations are transmitted from sperm to carrier daughters and from eggs to carrier daughters and affected sons, which appear as sporadic cases. Therefore, the proportion of cases that are sporadic depends on the relative mutation rates in egg and sperm. At the only sex-linked locus so far studied, mutation rates are substantially the same in the two sexes. This result is based on careful medical study of very large numbers of X chromosomes in different genetic backgrounds. It may be contrasted with the impracticability of similar investigations in other mammals, and with mutually contradictory Drosophila studies in which sex-linked mutations in eggs of selected genotypes are detected as sporadic cases according to their viability and the acuity of the observer. This is one instance where human genetics is more precise than the genetics of other organisms.

Discrimination of Genetic Entities

Every science is concerned with the unity of its elements. Resolution of loci and alleles has the same fundamental importance to the geneticist as purity to the chemist, recognition of clinical entities to the physician, or fractionation of antibodies to the immunologist. Segregation analysis and linkage provide the ultimate criteria of genetic unity, but useful phenotypic guides are furnished by chemistry, immunology, and medicine Sometimes a single diagnostic criterion is adequate to resolve phenotypes, but in more difficult cases several criteria must be used in combination. For example, sex-linked and autosomal gargoylism differ in the frequencies of corneal clouding,

TABLE 2 The Expected Number of Affected Persons When the Segregation Frequency Is ½, Selection Is Truncate, and There Are No Sporadic Cases. 600 Sibships of Size 2 and 700 Sibships of Size 3

Sibship size s	Cases r	Number Expected	Score for $p = \frac{1}{2}$ u_p	Variance of score k_{pp}
2	1	400	-1.3333	3.556
	2	200	2.6667	3.556
		600	0	2134
3	1	300	-2.8571	6.122
	2	300	1.1429	9.122
	3	100	5.1429	9.122
		700	0	5485
Total		1300	0	7619

Number of sibs examined = 2(600) + 3(700) = 3300
Expected number of affected persons = 1(700) + 2(500) + 3(100) = 2000

Naive estimate: $p^* = \frac{2000}{3300} = .606$

Excluding index cases: $p^* = \frac{2000 - 1300}{3300 - 1300} = .350$

Estimate from scores for truncate selection. $p^* = \frac{1}{2} + 0/7619 = .5000$

$$\sigma_{p^*} = 1/\sqrt{7619} = .0115$$

one of them affected, and assume that selection is truncate, that there are no sporadic cases, and that the segregation frequency is ½. We expect $(\frac{1}{2})^2/[1 - (\frac{1}{2})^2] = 1/3$ of the families with two children to have both affected, and the remainder to have an isolated case. Similarly, in families with three children, we expect 3/7 to have an isolated case, 3/7 to have two affected children, and 1/7 to have all three children affected. For purposes of illustration, and to keep the expected number of families integral, we may suppose the data consist of 600 families of two children and 700 families of three children. Each possible outcome is assigned a score for the segregation frequency, calculated on the above assumption that $p = \frac{1}{2}$, $\pi = 1$, and $x = 0$. For each family size, the scores sum to zero except for round-

may conceive of every mating that has produced an affected child as being of either of two types: a low-risk type, in which the probability of an affected child is so small that the risk for two or more affected children is negligible; and a high-risk type, in which the chance for other cases after the first is appreciable. The risk in high-risk families is called the *segregation frequency*. The low-risk (*sporadic*) type may be composed of mutants, phenocopies, rare instances of penetrance of recessive genes in heterozygotes, and more complex cases. Since in general we cannot tell whether an isolated case is sporadic or not, we designate by x the probability that an affected person in the population be sporadic, and by p the segregation frequency. Then we can estimate and test hypotheses about x and p, and derive the probability that an isolated case be sporadic which is $x/\{x + (1-x)\ (1-p)^S\}$ for an isolated case with S sibs. For dominant and sex-linked genes, the estimate of x leads to estimation of the mutation rate by the semidirect method.

The most familiar values of the segregation frequency are ½ and ¼, but proportions modified by differential mortality and partial manifestation are also included. For example, if ¼ of the children from a particular mating type are expected to be of a certain genotype, but only 80 per cent of them develop a characteristic abnormal phenotype, we shall say that the segregation frequency is ¼ (.80) = .20. This concept of incomplete penetrance is liable to abuse by uncritical workers, but under certain conditions it is a valid and fruitful hypothesis. These conditions include dominant genes whose characteristic manifestations occasionally "skip" a generation, and rare diseases with delayed onset, for which the penetrance may be calculated from the distributions of age at onset and the ages of family members, without any genetic assumptions.

In the past, simultaneous estimation of the parameters r, p, and x has presented considerable difficulties. Typically, many more families are available for segregation analysis than for linkage tests, so that large-sample theory need not be avoided. The most rigorous, efficient, and convenient analysis is by means of maximum likelihood scores and their variances, which provide tests of hypotheses, estimates, and standard errors for all parameters (Morton, 1958, 1959). Complicated situations are handled easily by means of available programs for electronic computers, permitting a modern geneticist to analyze, with precision, phenomena that would be hopelessly confused without these computational aids.

Consider a sample of families with two or three children, at least

TABLE 2 The Expected Number of Affected Persons When the Segregation Frequency Is ½, Selection Is Truncate, and There Are No Sporadic Cases 600 Sibships of Size 2 and 700 Sibships of Size 3

Sibship size s	Cases r	Number Expected	Score for $p = \frac{1}{2}$ u_p	Variance of score k_{pp}
2	1	400	-1.3333	3.556
	2	200	2.6667	3.556
		600	0	2134
3	1	300	-2.8571	6.122
	2	300	1.1429	9.122
	3	100	5.1429	9.122
		700	0	5485
Total		1300	0	7619

Number of sibs examined = 2(600) + 3(700) = 3300
Expected number of affected persons = 1(700) + 2(500) + 3(100) = 2000

Naive estimate: $p^* = \frac{2000}{3300} = .606$

Excluding index cases: $p^* = \frac{2000 - 1300}{3300 - 1300} = .350$

Estimate from scores for truncate selection: $p^* = \frac{1}{2} + 0/7619 = .5000$

$\sigma_{p^*} = 1/\sqrt{7619} = 0115$

one of them affected, and assume that selection is truncate, that there are no sporadic cases, and that the segregation frequency is ½. We expect $(½)^2/[1 - (½)^2] = 1/3$ of the families with two children to have both affected, and the remainder to have an isolated case. Similarly, in families with three children, we expect 3/7 to have an isolated case, 3/7 to have two affected children, and 1/7 to have all three children affected For purposes of illustration, and to keep the expected number of families integral, we may suppose the data consist of 600 families of two children and 700 families of three children. Each possible outcome is assigned a score for the segregation frequency, calculated on the above assumption that $p = ½$, $\pi = 1$, and $x = 0$ For each family size, the scores sum to zero except for round-

ing errors. Both the scores and their variances are additive over all family sizes, and lead by iteration from an incorrect hypothesis to the maximum likelihood solution. The sum of the variances of the scores is the amount of information about the segregation frequency, and the reciprocal of this sum is the variance of the estimate.

Let us examine a few possible estimates of the segregation frequency. A naïve estimate may be obtained by dividing 2000, the number of affected children, by 3300, the total number of children. This estimates the segregation frequency as $p^* = .606$, which is too large because it ignores the fact that the families were selected because they had at least one affected child. At a slightly more sophisticated level, we might deduct one proband from each sibship, on the argument that one affected person is a necessary and sufficient condition for selection of the family. Since there are 1300 families, we obtain $p^* = (2000 - 1300)/(3300 - 1300) = .350$, which is too small because this overcorrects for the sampling bias. Finally, we may use the maximum likelihood estimate, which, like Goldilocks' third choice, is just right.

TABLE 3. The Expected Number of Affected Persons When the Segregation Frequency is $p = 1/4$, Selection Is Multiple with $\pi = 1/4$, and the Proportion of Cases That Are Sporadic Is $x = 1/2$. 945 Sibships of Size 2 and 1489 Sibships of Size 3

Sibship size s	Cases r	Number Expected	u_p	k_{pp}	u_x	k_{xx}
2	1	840	-.5079	2.064	.2540	.5160
	2	105	4.0635	2.064	-2.0317	.5160
		945	0	1950	0	488
3	1	1200	-.8364	2.905	.4969	1.025
	2	252	2.7903	6.080	-2.0631	1.025
	3	37	8.1236	6.080	-2.0631	1.025
		1489	0	5243	0	1526
Total		2434	0	7193	0	2014

$$p^* = \tfrac{1}{4} + 0/7193 = .2500$$
$$\sigma_{p^*} = 1/\sqrt{7193} = .0118$$
$$x^* = \tfrac{1}{2} + 0/2014 = .5000$$
$$\sigma_{x^*} = 1/\sqrt{2014} = .0223$$

TABLE 4 The Expected Number of Ascertainments and Probands When the Segregation Frequency Is 1/4, *Selection Is Multiple With* $\pi = 1/4$, and the Proportion of Cases That Are Sporadic Is 1/2. 945 Sibships of Size 2 and 1489 Sibships of Size 3

Ascertainments per proband	Expected Number	Score for $\pi = \frac{1}{4}$ u_π	Variance of score $k_{\pi\pi}$
t			3.3853
1	2101	-.69859	3.3853
2	302	3.93616	3.3853
3	29	8.57091	3.3853
4	2	13.20565	
Total	2434	0	8240

Affected r	Probands a	Expected Number	u_π	$k_{\pi\pi}$
1	1	2040	0	0
2	1	306	-0.76190	3.483
	2	51	4.57143	3.483
		357	0	1243
3	1	21	-1.58559	6.788
	2	9	3.74775	9.348
	3	1	9.08108	9.348
		37	0	277
Total		2434	0	1520

$$\pi^* = \tfrac{1}{4} + 0/9760 = .2500$$

$$\sigma_{\pi^*} = 1/\sqrt{9760} = .0101$$

Since this model fits extremely closely, we have no reason to be dissatisfied with it. However, if the fit were poor, judged by the ratio of the score to its standard error, we would not know from this analysis whether the deviation lay in π, p, x, or in two or more of these simultaneously. To clarify this we must consider the scores for all parameters. Table 3 illustrates a problem of this kind, where the expected numbers are calculated on the hypothesis that $p = 1/4, \pi = 1/4$, and $x = 1/2$, the total numbers being chosen, as before, to give

integral expectations. The scores for p and x on this hypothesis sum to zero for each family size and are additive over all families. The segregation data give little information about π, but this can be estimated independently from the distributions of ascertainments and probands. If several independent methods of ascertainment are used, a proband may be directly ascertained once, twice, or more times, each of these possibilities being scored for π. In addition, the distribution of probands among affected sibs can be scored. In this example it will be noted that ascertainments give more than five times as much information as probands about the ascertainment probability, largely because the proband distribution for isolated cases is uninformative. Human geneticists have learned only recently to appreciate the large amount of information that may be discarded when the number of ascertainments of probands is not recorded.

This kind of detailed analysis has been applied so far only to muscular dystrophy, deaf-mutism, and the ABO and MN blood factors. Morton and Chung (1958) found that dominant and sex-linked types of muscular dystrophy conform closely to genetic expectation, *even in regard to the proportions of sporadic cases and affected uncles* in sex-linkage, and that the limb-girdle type of dystrophy includes both sporadic and fully penetrant, recessive cases. It could not be determined whether the sporadic cases were penetrant heterozygotes, phenocopies, or were caused by some other mechanism. Chung, Robison, and Morton (in press) found that all mating types for congenital deaf-mutism were mixtures of high-risk and low-risk families, the majority of cases being due to fully penetrant recessive genes and most of the remainder to dominant mutants and dominant genes transmitted from affected persons. The sporadic cases not accounted for by these mechanisms comprise only 9 per cent of all congenital deaf-mutes in the population studied. Papers to be published soon will demonstrate that the excess of MN children observed by many investigators in the progeny of MN mothers is not due to technical errors, by showing that the frequency of error estimated from nonsegregating families is far too small to account for the MN excess. There is a deficiency of ABO-incompatible children with increasing parity, this selection being balanced primarily by an advantage of AO fetuses in compatible matings.

The limited use made of these methods so far is due to their novelty and the unfamiliarity of many geneticists with the power and simplicity of maximum-likelihood scoring procedures. It is the best pro-

cedure for all segregation analyses, especially of complex or extensive data

CONCORDANCE IN TWINS

Since the time of Francis Galton, twin studies have seemed to some students to hold forth the promise of separating the contributions made to family resemblance by heredity and environment. An entire journal is devoted to this so-called "twin-method," but the assumptions required for estimation of heritability from twin studies are so little subject to proof that they have taken on the character of articles of faith, to which few of the younger geneticists adhere. Nevertheless, concordance of twins is often a useful datum, especially when the cause of discordance in monozygotic twins can be identified. Even a single instance of discordance of monozygotic twins is critical evidence against the hypothesis that incomplete penetrance is due strictly to genetic modifiers of a major gene. Monozygotic twins provide an upper estimate of penetrance and valuable experimental material on the effects of environmental differences, while comparison of the concordance of dizygotic twins and ordinary siblings gives evidence of transient uterine and postnatal environmental differences within families. In conjunction with other family data, twin concordances are of circumstantial and qualitative value in assessing the role of genetic factors.

To obtain comparability with segregation frequencies and empirical risks, concordance may be defined in a somewhat novel way as the probability that a twin be affected if the cotwin is. When we attempt to estimate concordance, a difficulty becomes apparent. We can determine the ascertainment probability π from data on probands and *ascertainments;* that twin investigators rarely do this, or even record these data, is in principle irrelevant, although it reduces the value of most twin studies in the literature. But when we come to estimate the concordance in high-risk twinships (p) and the frequency of sporadic cases (x), we find that this is impossible, since our only datum is the observed proportion of concordant twins, which is formally the same as the proportion of familial cases in sibships of size 2. Knowing x, we can estimate p, or knowing p we can estimate x, but we cannot determine both simultaneously. Without a segregation analysis of other kinds of family data, our only recourse is to assume that there are no sporadic cases.

The consequences of such an assumption are best appreciated from an example. Limb-girdle muscular dystrophy occurs sporadically in

about 40 per cent of cases and is due to highly penetrant recessive genes in the remainder. Accordingly, we should expect the concordance for monozygotic twins to be at least $p = .60$, but for dizygotics to be only $p = (.60)\ (1/4) = .15$, or less, depending on the age at examination. From these data we would correctly conclude that genetic factors are important in this type of muscular dystrophy, although with less assurance than by analysis of segregating families. However, we would be unable to demonstrate from twin concordance the mixture of recessive and sporadic cases which is so easily shown by family analysis. Not only are twin studies incapable of elucidating complex genetic situations, but they are of limited value in simple cases.

Empirical Risks

Progress in genetics has been dependent on discontinuous characters, the discontinuity being determined in any particular experiment by a small number of genes. Those who urge human geneticists to attack multifactorial conditions, because of their practical importance, fail to realize how little progress has been made in critical biometrical analysis with experimental organisms, and how much less suitable man is for such studies than plots of barley or corn.

Although traits determined by many genes, not individually recognizable, are unfavorable for genetic analysis, the medical importance of such conditions as breast cancer, hypertension, and amentia has led to preliminary investigations of the empirical risks to which relatives of probands are subject. These studies necessarily give much less accurate information than analyses of simpler conditions, due to ignorance of the responsible genetic mechanisms and to heterogeneity of the material. Often the sampling of probands is irregular, with overrepresentation of high-risk families. Commonly the method of ascertainment is ignored. Because of variations in sampling and diagnosis, even careful and enormously laborious studies often give only rough agreement.

While some of these difficulties are inherent in empirical risks, others can be avoided. Ascertainment of probands can be defined rigorously to permit valid and efficient analysis. Heterogeneity of the material can be reduced by discrimination of genetic entities and recognition of acquired cases. However, at some time in the analysis, further splitting of categories becomes impractical, and it is then necessary to choose the best method of analysis for data that may still be heterogeneous. Traditionally it has been assumed at this juncture

that the risk is uniform among all families within a category. In terms of our segregation analysis, the probability of the data is expressed as a function of p, the empirical risk, and π, the ascertainment probability, with no sporadic cases ($x = 0$). However, the assumption of sporadic cases has proved so fruitful for analysis of simple genetic situations, that we may wonder whether it would not be desirable to introduce the same concept into empirical risks. That this is desirable is apparent from the consideration that a model with three parameters should fit empirical data better than a model with only two parameters. The assumption of sporadic cases must also be an advantage as a working hypothesis, since it may lead to recognition of heterogeneity in data hitherto regarded as homogeneous and may even show that the risk in high-risk families is great enough to suggest a simple genetic hypothesis. Finally, the concept of sporadic cases is forced on us by mutations, instances of heterozygous expression of recessive genes, and causes of acquired cases that must usually be nonrecurrent in sibs, such as intra-uterine infection and birth trauma in mental deficiency, infections of infancy in deaf-mutism, or carcinogenic radiation in cancer.

The gross errors that may be committed when sporadic cases are ignored can be illustrated by sex-linked muscular dystrophy. If the mother is a carrier, we expect 50 per cent of her sons to be affected and 50 per cent of her daughters to be carriers. However, because many isolated cases are mutations, the risk that the brother of an isolated case be affected or his sister be a carrier may be very much smaller than this. For example, if the proband has four

TABLE 5 The Upper Limit to the Probability That a Brother of an Isolated Case of Sex-linked Muscular Dystrophy Be Affected or That a Sister Be a Carrier, if There Are S Normal Brothers and s Normal Maternal Uncles

s uncles	S brothers				
	0	2	4	6	∞
0	.33	.20	.09	.04	0
2	.28	.15	.06	.02	0
∞	.25	.15	.05	.02	0

normal brothers and two normal maternal uncles, the risk for a subsequent sibling is less than 6 per cent. Here we are alerted to sporadic cases because genetic theory predicts their occurrence. We have no reason to suppose that sporadic cases are less important in more complex conditions.

To a geneticist like myself it is remarkable, and to an enthusiast of "genetic counseling" it should be profoundly disturbing, that not only do we not know what proportion of cases of cleft palate, congenital heart disease, mental defect, or breast cancer is sporadic, but the question has not even been clearly formulated in the empirical risk studies that have been carried out. For many conditions it seems likely that "genetic counselors" are systematically underestimating empirical risks in families with two or more affected, and overestimating them in families with only one affected. Fortunately, this error can be avoided by using the general form of the segregation analysis, with no assumed restriction on the frequency of sporadic cases, and it will be interesting to see how materially this contributes to the specification of empirical risks in the future.

Although increased knowledge of empirical risks is to be desired, there are so few human geneticists that we do not need to justify our preoccupation with more academic and technical questions. It is less easy to explain our slowness to develop adequate methods of analysis and especially to familiarize other practitioners of human genetics with them. Few studies of empirical risks, concordance in twins, or even formal genetic analyses, attain the power and precision of which they are capable. They provide a shaky basis for theory or application. The methods described in this paper and others recently developed will help to provide a better foundation for human genetics.

SUMMARY

Progress in human genetics may be divided into three stages: search for Mendelian inheritance; precise analysis under restrictive assumptions; and generalization of the conditions under which similar phenomena, genetic or environmental, can be incisively resolved. These successive developments are exemplified by analysis of linkage, partial sex linkage, and mutation.

An important class of problems includes segregation ratios, empirical risks, and concordance in twins. The underlying assumptions are formally the same, and therefore the best methods of analysis are identical. In the most general situation so far investigated, the funda-

mental equations are functions of three parameters. One (π) expresses the completeness of sampling in terms of the probability that an affected person in the population be investigated. Another (x) represents sporadic cases, of whatever origin, the risk for repetition of which is negligible in a small family group. The third parameter (p) gives the probability of being affected in a high-risk family. The actual number of persons affected in such a family is determined by chance, with a specified risk of no affected individuals or only an isolated case. In any particular instance we may not be able to tell whether an isolated case is sporadic, but we can determine rigorously what the probabilities are.

Interpretation of genetic data in terms of this model is illustrated for genetic factors, empirical risks, and concordance in twins.

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Genetic Considerations in Human Breast and Gastric Cancer

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BREAST CANCER

The evidence to be presented, that human breast cancer depends in part upon genetic factors, deals with the number of breast cancers in the relatives of three sets of women known as probands. The first group is composed of 295 women with pathologically diagnosed breast cancer, called P1; the second group, 300 patients with some cancer other than breast, called P2; and the third group, 246 women who had no known cancer at the time of interview, called P3. The same questions were put to all of these probands, and the relatives about whom information was sought were grandparents, parents, aunts, uncles, sibs (meaning brothers and sisters), children, and first cousins. Every item of information concerning these relatives in the three populations was then checked by writing to all of the living relatives to confirm or correct the data given by the patient about them and other relatives, and by obtaining as far as possible all death certificates of the deceased relatives. Thus, many relatives forgotten by the proband, and many items about other relatives, were brought to light by this investigation, and the problem of better recall of relatives with breast cancer by a woman with breast cancer was eliminated.

Co-operation by the proband was essential. It is held that a woman with breast cancer will co-operate better because she has a large number of breast cancers in her family. This was not the case in this study A total of 196, or 66 per cent of the women, co-operated, but reported

no relatives with breast cancer. Ninety-nine, or 34 per cent, of those co-operating reported breast cancer in their relatives, but in 59 instances these cases could not be verified The accuracy of their reporting was low, especially in those instances in which the proband reported breast cancer in other relatives. Only 23 of the 99 gave accurate reports; 17 others reported breast cancers which could not be verified, and failed to report other instances of breast cancer which were found. Thirty-three of those reporting no breast cancer were found to have cases of breast cancer in their relatives.

P2 probands were taken to determine whether factors inducing cancer in families were site specific, or were merely for cancer in general. If they were site specific, neither P2 nor P3 relatives should have any more breast cancer than the general population, while P1 relatives should have an increased number. If the factors concerned cancer in general, both P1 and P2 relatives should have an excess of breast cancer, while P3 relatives should not.

No case of breast cancer reported in relatives was coded as an actual case unless a death certificate was found giving breast cancer as the cause of death Two methods were used in assessing the number of breast cancers expected in these three populations. The first was the age-specific proportionate death rates based on Ohio data from 1910 through 1952 which, for the sake of verity, will hereafter be referred to as proportionate death rates The second was the age-specific mortality rates from breast cancer for the whole United States, to be referred to as mortality rates

Proportionate death rates from breast cancer have changed materially over the last 50 years In order to use this method, the average number of deaths and the average number of breast cancer deaths in white women in Ohio were computed by quinquennial age groups beginning with 30 to 34 and ending with 90+, for each of the decades from 1910 through 1952. The rate for 1910 alone was used for those deaths occurring before 1910 It was too high, but was the only one available. Mortality rates, based upon the number of deaths from breast cancer among 100,000 living women at that age, have increased, but only to a limited degree, so that the 1940 mortality rates were used in the second method. The first rate expresses the probability that a death will be due to breast cancer; whereas the second rate expresses the probability that one will die in a given age range and that the death will be due to breast cancer.

In this study, both methods showed that the relatives of women with breast cancer had significantly more breast cancer than either

the women of the general population, or the relatives of the two sets of control probands. These latter relatives, moreover, had approximately the same amount of breast cancer as the women of similar age distribution in the general population. This shows that the two sets of control relatives (P2 and P3) were indeed representative of the general population, while the P1 relatives were not.

It was shown by the use of proportionate death rates that the relatives of P1 had about the same amount of other kinds of cancer as the general population of Ohio showed. Therefore, the excess of breast cancer could not be attributed to a marked decrease in number of other cancers.

TABLE 1. Breast Cancer Frequency in P1

Relatives	No Dead	No Breast Cancers Observed	No. Breast Cancers Expected on Proportionate Death Rates	No Relatives Living and Dead	No Breast Cancers Expected on Mortality Rates
Grandmothers	297	17	4.5	301	7.4
Mothers	213	11	4.6	255	5.6
Aunts	682	43	14.5	891	20 1
Sisters	132	14	4.6	498	4 8
Total	1324	85	28 2	1918	37 9
			$\chi^2 = 116.9$ P < .001		$\chi^2 = 59.60$ P < 001

The number of verified cancers in the relatives of P1 is shown in Table 1. For proportionate death rates, only the deceased members can be used; but for the mortality rates, both living and deceased are used, with the various relatives leaving the table either at the age attained when they died or when they were last contacted by the author. Only those breast cancers occurring in the deceased, however, are counted in both methods.

Note that in each of the relationships shown, the number of breast cancers observed was greater than the number expected by either method. A total of 85 breast cancers was found, but only 28.2 and 37.9, respectively, were expected by the two methods. The P value is set at .001, but is far less than that. No table of χ^2 values was available beyond this limit. The mortality rate method yields a higher

expectation than the proportionate death rate method except in the case of sisters, where the two values are almost identical This is because the mortality rates were based on years much later than those in which many of the relatives except sisters died; whereas the proportionate death rates were based on years in which the relatives died, except for those dying before 1910. Breast cancer occurred two and one-half to three times as often in P1 relatives as in the general population of the same age and decade of death distribution in Ohio. The probability is very small that this difference was due to chance sampling.

TABLE 2 Breast Cancer Frequency in P2

Relatives	No Dead	No Breast Cancers Observed	No Breast Cancers Expected on Proportionate Death Rates	No Relatives Living and Dead	No Breast Cancers Expected on Mortality Rates
Grandmothers	283	2	4 4	286	6 7
Mothers	203	4	3 9	257	5 9
Aunts	636	11	12 9	858	19.4
Sisters	162	6	5 2	524	5.9
Total	1284	23	26 4	1925	37 9
			$\chi^2 = 23$ P > 50		$\chi^2 = 5\ 97$ P > 01

This table presents corresponding figures for the relatives of control probands with cancer other than breast. Twenty-three breast cancers were found, and 26.4 and 37 9 were expected by the two methods The first difference is not significant, although the second is at the 2 per cent level. Again the expectations for sisters were almost identical with each other, and also with the number found. In the other relatives the mortality rate again gave higher figures than the proportionate death rates, but again the former rates were too high for the periods of time in which these relatives were living.

P1 relatives *can* be compared directly with P2 relatives since the age, decade-of-death and relationship distribution of the two groups is very close, taking each relationship in turn. If the two sets of relatives are compared directly, P2 relatives should have had proportion-

the women of the general population, or the relatives of the two sets of control probands. These latter relatives, moreover, had approximately the same amount of breast cancer as the women of similar age distribution in the general population. This shows that the two sets of control relatives (P2 and P3) were indeed representative of the general population, while the P1 relatives were not.

It was shown by the use of proportionate death rates that the relatives of P1 had about the same amount of other kinds of cancer as the general population of Ohio showed. Therefore, the excess of breast cancer could not be attributed to a marked decrease in number of other cancers.

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Sisters	132	14	4.6	498	4.8
Total	1324	85	28.2	1948	37.9
			$\chi^2 = 116.9$ $P < .001$		$\chi^2 = 59.69$ $P < .001$

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TABLE 2. Breast Cancer Frequency in P2

Relatives	No Dead	No Breast Cancers Observed	No Breast Cancers Expected on Proportionate Death Rates	No. Relatives Living and Dead	No Breast Cancers Expected on Mortality Rates
Grandmothers	283	2	4 4	286	6 7
Mothers	203	4	3 9	257	5 9
Aunts	636	11	12 9	858	19.4
Sisters	162	6	5 2	524	5 9
Total	1284	23	26 4	1925	37 9
			$\chi^2 = .23$ $P > 50$		$\chi^2 = 5\ 97$ $P > 01$

This table presents corresponding figures for the relatives of control probands with cancer other than breast. Twenty-three breast cancers were found, and 26 4 and 37 9 were expected by the two methods. The first difference is not significant, although the second is at the 2 per cent level. Again the expectations for sisters were almost identical with each other, and also with the number found. In the other relatives the mortality rate again gave higher figures than the proportionate death rates, but again the former rates were too high for the periods of time in which these relatives were living.

P1 relatives can be compared directly with P2 relatives since the age, decade-of-death and relationship distribution of the two groups is very close, taking each relationship in turn. If the two sets of relatives are compared directly, P2 relatives should have had proportion-

the women of the general population, or the relatives of the two sets of control probands. These latter relatives, moreover, had approximately the same amount of breast cancer as the women of similar age distribution in the general population. This shows that the two sets of control relatives (P2 and P3) were indeed representative of the general population, while the P1 relatives were not.

It was shown by the use of proportionate death rates that the relatives of P1 had about the same amount of other kinds of cancer as *the general population of Ohio showed. Therefore, the excess of* breast cancer could not be attributed to a marked decrease in number of other cancers.

TABLE 1. Breast Cancer Frequency in P1

Relatives	No. Dead	No Breast Cancers Observed	No Breast Cancers Expected on Proportionate Death Rates	No. Relatives Living and Dead	No Breast Cancers Expected on Mortality Rates
Grandmothers	297	17	4 5	301	7.4
Mothers	213	11	4.6	255	5 6
Aunts	682	43	14 5	891	20 1
Sisters	132	14	4.6	498	4 8
Total	1324	85	28.2	1918	37.9
			$\chi^2 = 116\ 9$ P < .001		$\chi^2 = 59\ 69$ P < 001

The number of verified cancers in the relatives of P1 is shown in Table 1. For proportionate death rates, only the deceased members can be used; but for the mortality rates, both living and deceased are used, with the various relatives leaving the table either at the age attained when they died or when they were last contacted by the author. Only those breast cancers occurring in the deceased, however, are counted in both methods.

Note that in each of the relationships shown, the number of breast cancers observed was greater than the number expected by either method. A total of 85 breast cancers was found, but only 28.2 and 37.9, respectively, were expected by the two methods. The P value is set at .001, but is far less than that. No table of χ^2 values was available beyond this limit. The mortality rate method yields a higher

ish study. His estimate of the amount of breast cancer expected in the control group and breast cancer relatives was based on modern rates, not on rates existing at the time the relatives were living. This is the objection to the mortality rates for 1940 in this study. Women who died fifty years ago at age 60 probably had very different childbearing and nursing histories from those dying at age 60 in 1959. The proportionate death rate method shows that P2 and P3 had breast cancer in numbers closely agreeing with the population, while P1 had significantly more.

This increase might be dependent upon genetic or environmental factors or upon a combination of the two. It might be dependent upon a bias engendered by the method of collection of data. Evidence for or against these various interpretations will be discussed

First consider environment, especially the milk agent. If this is responsible, those females who are related to the breast cancer proband by way of an unbroken line of females through whom a milk virus could be transmitted should have a significantly higher number of breast cancers than females related through a male. Paternal grandmothers and aunts can be compared with their counterparts on the maternal side for number of breast cancers P1 paternal grandmothers were found to have an age distribution very similar to that of P1 maternal grandmothers, and when compared directly for number of breast cancers were so similar that the probability of finding greater difference between them was greater than 70 The same similarity in age distribution and number of breast cancers was found when paternal and maternal aunts were compared. This close similarity argues not only against the presence of a milk factor but also against other environmental agents playing a predominant role, since the genetic component is equal from both parents except in the case of sex-linked traits, while the environmental influences are not likely to be so conspicuously similar.

Is the increased amount of breast cancer in relatives of breast cancer probands caused by a bias inherent in the method of collecting data? Theoretically, one is likely to find a family, in which two members have the trait being studied, twice as often as if the family had but one such affected member, since the family could be ascertained through either of the affected persons This increased probability of finding the family with multiple cases would be experienced both when the trait is caused by genetic factors and when it is distributed at random as a nongenetic trait through the population The commoner the trait, the more likely one is to find families with multiple

ately 82.4 breast cancers in place of 23, if they were to be comparable with P1.

Whatever factors are responsible for inducing breast cancer, they must be site specific, since P2 relatives did not have any more breast cancer than the general population, and P1 relatives had significantly more.

TABLE 3 Breast Cancer Frequency in P3

Relatives	No Dead	No Breast Cancers Observed	No Breast Cancers Expected on Proportionate Death Rates	No Relatives Living and Dead	No Breast Cancers Expected on Mortality Rates
Grandmothers	302	3	4 7	306	7.6
Mothers	158	3	3.5	221	4.6
Aunts	497	11	10.5	753	17.1
Sisters	74	5	2.5	350	3.4
Total	1031	22	21.2	1630	32.7
			$\chi^2 = .03$ $P > 80$		$\chi^2 = 3.57$ $P > .05$

Table 3 gives data on the relatives of women who had no signs of cancer at the time of the interview The number of breast cancers observed and the number expected are very close with the proportionate death rate method (22 and 21.2), while the number expected when the mortality rates are used differs more widely, although the difference is not significant. P1 and P3 relatives in corresponding relationships had closely similar age and decade-of-death distributions. Before they can be compared directly, P3 relatives must correspond proportionately to the P1 group. This raises the number of breast cancers observed in P3 from 22 to 24 2. On the basis of the size of group they should have had 66 breast cancers in place of 24.2 if they had breast cancer as often as P1 relatives As in the case of P1 and P2, P1 relatives had almost three times as much breast cancer as the P3 control population, and as the general population of Ohio had

The criticism might be made at this point that the two control populations had too little breast cancer on the basis of the mortality rates, thus explaining the difference between P1 in the one case and P2 and P3 in the other. Busk made this objection to Jacobsen's Dan-

respectively, were found with breast cancer when the expectation was but .5. Here the difference is not significant. It appears that neither the chance distribution of the trait in the population nor the probability of finding families with multiple cases with undue frequency is enough to account for this excess of breast cancers in P1 relatives.

There remains one factor which has been found to be associated with differences in risk of breast cancer, and that is the marital state. All workers have agreed that single women have more breast cancer than married women have. The present study showed that single women have no more breast cancer than have childless married women of the same age distribution. It appears to be the lack of offspring or some associated factor as yet not determined with certainty, rather than the lack of a husband, which is the cause. Even here, the physiological factor of nulliparity appears to operate differentially upon different genetic backgrounds.

TABLE 5 Percentage of Breast Cancer Correlated with Parity and Genetic Background

No children per individual	0	1, 2	3, 4	5, 6	7+
No P1 relatives	135	241	280	239	307
No with breast cancer	20	17	16	13	18
% with breast cancer	14 8	7 0	5 7	5 4	5 8
No controls (P2 and P3 relatives)	241	438	542	413	596
No with breast cancer	12	13	12	4	7
% with breast cancer	5 0	3 0	2 2	1 0	1 2

Age distribution in each parity grouping is very similar for P1 and controls

The relatives of P1 have been grouped into those with no children, with one and two, three and four, etc. P2 and P3 relatives have been similarly grouped together under the heading of controls. The age distribution of P1 and controls in each parity grouping is similar, so that direct comparisons can be made.

The P1 nulliparae had twice as much breast cancer as P1 relatives with one and two children, thus showing the effect of this physiological factor in inducing breast cancer. Similarly, nulliparous controls had more breast cancer than control women with one and two

cases. Breast cancer is not a very common trait, however, and the probability of finding two sisters alive with it is not great. The living sisters of P1, P2, and P3 were compared for the numbers of breast cancers expected and observed to determine whether multiple cases were occurring too frequently to be explained on biased ascertainment. The results are shown in Table 4.

TABLE 4. Frequency of Breast Cancer in Living Sisters of P1, P2, and P3 Probands

Population Group	No Living Sisters	Average Age	No Breast Cancers Observed	No Breast Cancers Expected*	χ^2 (Yates Correction)
P1	374	54.6	10	.7	20 67 P < .001
P2	267	54 7	1	5	00 P > .99
P3	286	54 7	2	.5	2 00 P > .10

* See text for explanation

If correction is made for the greater risk of finding families with multiple cases, one should not find such families any more often than expected on the basis of frequency of the trait in the population, provided that the excess of families with multiple cases was indeed caused by the biased method of collecting the families. First, average ages of the living sisters in P1, P2, and P3 were found to be practically identical, as well as the age distribution also being similar. The average age was almost 55, and the risk of dying with breast cancer in 1940 at ages 55 through 59 was found to be almost 62. Since there are said to be three women living with the disease for every one who dies of it in a given time, the chance of finding a living woman with the disease at age 55 can be set at 186 per 100,000 living women Thus, one should find among 374 P1 living sisters 7 sisters with breast cancer. Actually, there were 10 living with the disease. But finding families with two affected is likely to occur twice as frequently as such families exist in the population. Therefore, there should be but five such families in the sample The probability of finding five living sisters with breast cancer when the expectation is only 7 is less than .001. Since these values are so small, χ^2 may not be applicable, but the conclusions are probably valid.

Similarly, in living sisters in P2 and P3, one and two living sisters,

age and sex distribution displayed, and that they were similar with each other in age distribution and decade of death. Hence, although the data of this study have not yet been subjected to a complete analysis by age-specific proportionate death and mortality rates, the tentative conclusions given here probably will not be modified to any extent when the more complete analysis is made.

Gastric cancer is not dealt with as easily as breast cancer. First, it is an internal cancer, the diagnosis of which was often missed in the past, so that many relatives who had died of cancer of the stomach were not so listed, thus minimizing the possible evidence of any genetic factors. The coding of the disease was also most inadequate, gastric cancer being grouped with cancer of the liver, both primary and secondary, before the year 1940. The method of analysis used here, therefore, is the estimation of the proportion of all cancers in each population which were gastric cancers. The pitfalls inherent in this method are fully appreciated, but when the populations compared are found to be comparable as far as age and decade-of-death distribution is concerned, and both are found to have amounts of all types of cancer comparable with the general population, the proportion of cancers that are gastric is a reliable method

In Ohio the males have almost twice as great a risk of developing gastric cancer as the females. The relatives of the gastric cancer and of the control probands were separated into males and females, and

TABLE 6 Frequency of Gastric Cancer in Male Relatives of 160 Gastric Cancer Patients

Relatives	No Gastric Cancers	No Rectal Cancers	No Other Cancers	% of Recorded Cancers that were Gastric Cancers
Grandfathers	3	1	0	75 0
Fathers	9	0	7	56 0
Uncles	10	3	10	43 5
Brothers	13	8	15	36 1
Totals	35	12	32	44 3
Expected rate of gastric cancer—14 6% in males over 40 years old.				
$\chi^2 = 5381$ P < .0001				
Male Cousins	18	8	44	25 7
$\chi^2 = 6\ 12$ P > 0 02				

children. But it will be noted also that when the environmental factor of nulliparity was kept constant, the genetic background was apparently able to induce almost three times as much breast cancer in P1 aunts and sisters as occurred in control aunts and sisters. Different workers may differ as to the degree of excess of breast cancer found in the relatives of the breast cancer probands. Not only may the genetic factors and background be different in the relatives, but the number of children per family and the amount of nursing per mother may differ widely, thus modifying the excess of breast cancer found.

It seems that one is justified in concluding that human breast cancer has a genetic basis, although the questions of how many genes are involved and the mode of transmission are left unanswered. Nor do I think that these questions will be answered by collecting pedigrees of individual families. Like most other cancers it appears to be influenced by factors other than genetic; in this instance, the only one we have any sure knowledge of is the role of childbearing. It is not clear through what channels this factor operates to suppress or enhance the genetic background, although extent of lactation is probably responsible to a large degree. Women who are closely related to women with breast cancer run a considerably higher risk of developing breast cancer than that experienced by women of similar age and physiological background not so related. Genetic background may be modified by age and number of children which the woman has borne.

Gastric Cancer

A similar study was done on 106 male and 54 female patients with pathologically diagnosed gastric cancer. Most of the patients had died before we could obtain the interview, but the family history was supplied by either a brother or a sister. The same care was taken in checking all information and in securing all death certificates as was shown in the breast cancer study. No death in a relative was coded as caused by gastric cancer unless this diagnosis was present on the death certificate. There were two controls for this group, the population data for the state of Ohio, and the selected group of families already intensively investigated in the breast cancer study as relatives of probands with breast cancer or with cancers other than stomach, and of probands with no cancer at all. In the breast cancer survey it had been shown already that these groups of relatives had about the same amount of cancer in general as the whole population of similar

age and sex distribution displayed, and that they were similar with each other in age distribution and decade of death. Hence, although the data of this study have not yet been subjected to a complete analysis by age-specific proportionate death and mortality rates, the tentative conclusions given here probably will not be modified to any extent when the more complete analysis is made.

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Grandfathers	3	1	0	75.0
Fathers	9	0	7	56.0
Uncles	10	3	10	43.5
Brothers	13	8	15	36.1
Totals	35	12	32	44.3
Expected rate of gastric cancer—14.6% in males over 40 years old				
χ^2 33.84 $P < 0.001$				
Male Cousins	18	8	44	25.7
χ^2 6.12 $P > 0.02$				

children. But it will be noted also that when the environmental factor of nulliparity was kept constant, the genetic background was apparently able to induce almost three times as much breast cancer in P1 aunts and sisters as occurred in control aunts and sisters. Different workers may differ as to the degree of excess of breast cancer found in the relatives of the breast cancer probands. Not only may the genetic factors and background be different in the relatives, but the number of children per family and the amount of nursing per mother may differ widely, thus modifying the excess of breast cancer found.

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cant excess of gastric cancers in females dying past age 40 in comparison with the number shown in the state as a whole, where only 8.7 per cent of females dying after age 40 had cancer of the stomach. The percentage of gastric cancers among all cancers for all female relatives exclusive of cousins was 29.6. The probability that this was caused by chance is small Female cousins also had more stomach cancer than expected, although the difference is not nearly so marked as in the other relatives.

TABLE 8 Frequency of Gastric Cancer in Male Relatives of Probands without Gastric Cancer

Relatives	No Gastric Cancers	No Gastric Cancers*	No Other Cancers	No Other Cancers*	% of Recorded Cancers that were Gastric Cancer	% of Recorded Cancers that were Gastric Cancer*
Grandfathers	28	9.7	43	14.8	39.4	
Fathers	19	26.3	53	73.4	26.4	
Uncles	81	42.7	190	100.2	29.9	
Brothers	13	37.8	64	186.1	16.9	
Totals	141	116.5	350	374.5	28.7	23.7%

* If the relatives occurred in same proportion as they occurred for gastric cancer probands in Table 6

The conclusion concerning the excess of gastric cancers also was checked against the control sample. In the Ohio statistics, the age groups cover 20-year periods, which provide far too large an age range to be satisfactory The relatives of gastric cancer probands and the relatives of the 1,033 families previously studied in which the proband did not have gastric cancer were divided into quinquennial age groups. They had been shown to be similar in age distribution within their small groups, as well as in decade-of-death distribution. When these relatives were arranged in a manner similar to those for the gastric cancer probands, it was found that they too had an excess of gastric cancer when compared with the data for the whole state. These relatives constituted a *better* and a more stringent criterion by which to judge the relatives of the gastric cancer probands.

In Table 8, the male relatives of probands with various types of cancer other than stomach and the male relatives of probands with no

into the various relationship groups studied. Cancer of the large bowel was noted frequently in the relatives of the gastric cancer probands, and one of the questions studied was whether it occurred more frequently than was expected on the basis of its distribution in the general population. If this occurred, it would suggest that some or all of the factors inducing gastric cancer also induced bowel cancer. The analysis to date has not shown cancer of the large bowel to be any more frequent in relatives of gastric cancer probands than would be expected on the basis of chance.

There were few cancers recorded in the grandfathers, but 75 per cent of those found were in the stomach. Similarly, 56 per cent of the cancers recorded in the fathers, 43.5 per cent of those in the uncles, and 36.1 per cent present in the brothers were gastric cancers. These were all males who had died past the age of 40. The total percentage was 44.3 in contrast to 14.6—the percentage of all cancers that were in the stomach in the male population of Ohio in 1952, where only males dying past age 40 were considered.

Cousins, who are the farthest removed genetically from the proband among the relatives considered, also had more gastric cancer than would be expected.

The death certificates of female relatives of gastric cancer probands then were inspected to see if the same excess was present in them. Here again the number of cancers was small, but there was a signifi-

TABLE 7 Frequency of Gastric Cancer in Female Relatives of Gastric Cancer Patients

Relatives	No. Gastric Cancers	No. Rectal Cancers	No. Other Cancers	% of Recorded Cancers that were Gastric Cancers
Grandmothers	7	1	9	41.0
Mothers	5	6	10	23.8
Aunts	12	3	23	31.6
Sisters	8	4	20	25.0
Totals	32	14	62	29.6
Expected rate of gastric cancer—8.7% in females over 40 years old				
$\chi^2 = 56.91$ $P < .0001$				
Female Cousins	15	8	49	20.8
$\chi^2 = 11.70$ $P < .001$				

GASTRIC CANCER

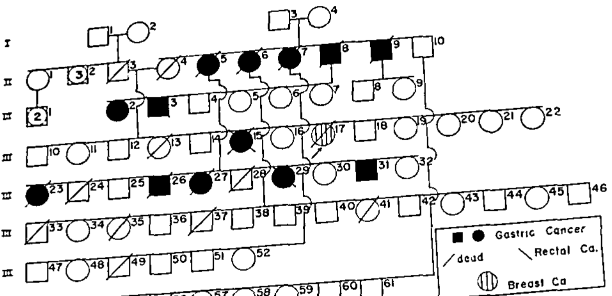


Figure 1 Family found in this study that had the greatest amount of gastric cancer in the family

cancer at all are listed, with the number of gastric and other kinds of cancer from which these relatives died. The percentage of cancers which were gastric was significantly higher for all relatives except brothers, when compared with 14.6—the percentage of cancers in the male population of Ohio that were gastric cancer; but also significantly lower than the percentage of gastric cancers found in Table 6 for relatives of gastric cancer patients. Before the totals in the two groups could be compared, however, both had to have the same proportion of the different relatives in each group. The proportions of relatives in each relationship in Table 8 were adjusted to correspond with the proportions found in Table 6. Thus, grandfathers in Table 6 had furnished 5 per cent of the total of all cancers in the table, but supplied 14.5 per cent of the total of all cancers in Table 8. When 5 per cent of the total in Table 8 was taken, it gave 24.6 for the number of cancers which the grandfathers should have had in place of the 71 cancers which they contributed to the total. The value of 24.6 cancers was then multiplied by the value of 39.4, which was the percentage of gastric cancers which these grandfathers showed. The result was 9.7, which represents the proportion of gastric cancers which these grandfathers of 1,033 control probands should have exhibited if the control grandfathers were contributing the same proportion of all cancers as the grandfathers of the gastric cancer probands were contributing.

The other relatives were treated similarly, and the proportionate

TABLE 9. Frequency of Gastric Cancer in Female Relatives of Probands without Gastric Cancer

Relatives	No. Gastric Cancers	No. Gastric Cancers*	No. Other Cancers	No. Other Cancers*	% of Recorded Cancers that were Gastric Cancer	% of Recorded Cancers that were Gastric Cancer*
Grandmothers	17	16.5	81	81.5	17.3	
Mothers	21	22.7	88	86.3	19.3	
Aunts	52	37.0	218	263.0	17.3	
Sisters	13	41.6	86	57.4	23.2	
Totals	103	117.8	503	488.2	17.0	19.4%

* If the relatives occurred in the same proportion as they occurred for gastric cancer probands in Table 7

that parent and child or sibs have the same condition far in excess of expectation.

One may conclude that gastric cancer, like breast cancer, is dependent in part upon genetic factors. Environmental influences are not as obvious as in breast cancer. There were few instances of the disease descending through three generations, so that the scattering of the disease through the pedigree would suggest more than one genetic factor involved.

CONCLUSION

In conclusion I would like to point out what I consider to be errors in Dr. Schull's viewpoint (see pp. 377 to 390, this volume). He has criticized the various studies on human populations on several grounds. First, they were not done on "definite or known" populations. Second, they all found about two and one-half to three times as much cancer in probands' relatives as compared with the relatives of the controls, no matter what type of control had been selected. Third, the same conclusions could have been arrived at through clinical experience, without these studies. Fourth, they had contributed nothing to the knowledge of genetics of cancer. In brief, they presented nothing but "chunks of data."

Let me take up these criticisms in reverse order. The beautifully executed studies of various cancers in inbred lines of mice, where matings can be controlled, have not yet pinpointed the number of genes responsible for various cancers, nor have they assigned any dominant or recessive role to these genes. They are just beginning to define the site of action of the genes. Why expect studies on heterogeneous human populations to solve what experimental work has left unanswered?

The conclusions that these particular forms of cancer—breast, gastric, and rectal—are in part genetically determined, could not have been and were not arrived at by clinical experience. The medical profession as a whole has been most reluctant to admit that heredity plays any role in cancer genesis. Most books dealing with cancer deny any role to heredity. Cowdry's book, *Cancer Cells* (1955), opens the chapter on heredity with the statment, "If there is one thing we know about cancer it is that it is not inherited." Most of the statements which one finds pertaining to inheritance of cancer have been come by through the questioning of patients by physicians concerning the presence of cancer in their relatives. The physician has to accept the statement of the patient without any attempt to

numbers of gastric cancers are shown in Table 8. There should have been a total of 116.5 gastric cancers in this control population instead of the 141 which were found. The two values from Table 6 and Table 8 can now be compared, and the χ^2 value of the difference was 14.76, with a P value of less than .001.

The female relatives of the 1,033 control families were similarly treated, and the results are shown in Table 9. In place of the 103 gastric cancers found, there should have been 117.8. Again the percentage of gastric cancers found in this control population is significantly higher than that found in the entire population, but significantly lower than that found in the relatives of gastric cancer probands. The χ^2 value of the difference was 5.68, with a P value a little greater than .01. The reason for the excess of gastric cancers in the control population doubtless resides in the fact that the control population was older than the state population, both of which were taken merely as being over 40. It is evident that both male and female relatives of probands with gastric cancer have gastric cancer in significantly greater numbers than corresponding relatives of similar age distribution of probands without gastric cancer.

The same statements about biased selection, co-operation of patients, and better recall that were enumerated under the discussion of breast cancer apply here. It was impossible to test the effect of biased ascertainment as in the case of breast cancer, where the frequency of the trait was determined in living sisters. There were no living sibs with gastric cancer, the disease claiming its victims so soon after diagnosis as to leave no sibs alive with it in this sample.

Figure 1 shows, however, that the family with the greatest amount of gastric cancer was not found because of the proband having gastric cancer, but was picked up because of the one person in the family with breast cancer. There are 14 cancers in this family, and 10 of them are gastric. Of the 19 persons who have died as adults, 8 have died of gastric cancer. This family strongly suggests a potent genetic factor or factors in gastric cancer.

One method of testing the relative effects of environmental and genetic factors is to compare the frequency with which two unrelated persons who share the same environment, such as husband and wife, develop gastric cancer, with the frequency with which two related persons who share the same environment, such as parent and child or two sibs, develop the disease The number of cases of spouses both of whom showed gastric cancer was in agreement with the expectation on the basis of population data, but it has already been shown

that parent and child or sibs have the same condition far in excess of expectation.

One may conclude that gastric cancer, like breast cancer, is dependent in part upon genetic factors. Environmental influences are not as obvious as in breast cancer. There were few instances of the disease descending through three generations, so that the scattering of the disease through the pedigree would suggest more than one genetic factor involved.

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The conclusions that these particular forms of cancer—breast, gastric, and rectal—are in part genetically determined, could not have been and were not arrived at by clinical experience. The medical profession as a whole has been most reluctant to admit that heredity plays any role in cancer genesis. Most books dealing with cancer deny any role to heredity. Cowdry's book, *Cancer Cells* (1955), opens the chapter on heredity with the statment, "If there is one thing we know about cancer it is that it is not inherited." Most of the statements which one finds pertaining to inheritance of cancer have been come by through the questioning of patients by physicians concerning the presence of cancer in their relatives. The physician has to accept the statement of the patient without any attempt to

check its accuracy. He does not have the time or the facility for verification. The physician's observations usually are phrased as follows: X percentage of women with breast cancer have relatives with breast cancer; while x, x-minus, or x-plus percentage of women without breast cancer have relatives with breast cancer. No attempt is made to see how many relatives of either group *could* have had breast cancer, their age distribution, etc. Thus, a control woman with 20 aunts could show more breast cancer among aunts than a woman with breast cancer who has no aunts. Or the conditions might be reversed, so that breast cancer was more prevalent among the aunts of women with breast cancer merely because they had more aunts. As an example of the type of statement which comes from clinical experience, let me cite the observation of one physician who was certain that a cause-and-effect relationship existed between cervical cancer and handling tobacco, because every case of cervical cancer in his practice had been in women who rolled tobacco leaves into cigars. Investigation on my part showed that every woman in that district was employed in a cigar factory; there was no other type of employment to be had. So pregnancies, broken legs, and need for glasses could have been attributed equally well by this man to rolling tobacco leaves into cigars. That a trained geneticist, versed in scientific methods, should state that such worthless impressions of clinicians are likely to give as valid results as those studies which have been undertaken on breast and gastric cancer by various workers even though they do not have the "defined" population of the epidemiologist, is amazing. "Chunks of data" they may be, but they are at least carefully collected and verified chunks, not the impressions of a busy clinician who does not even have "data."

The various studies have *not* all arrived at two and one-half to three times as much cancer in relatives of the probands as in relatives of controls. Jacobsen (1946) found 10 times as much; Vidabaek and Mosbech (1954) found four times as much; Anderson, Goodman, and Reed (1958) found less than two and one-half to three times as much; and Murphy (1952) found no significant difference in cervical cancer. It is just possible that two and one-half to three times may be the excess found in a heterogeneous population of human beings who in all probability carry genes for numerous kinds of cancer and who usually succumb to the type which shows first, either because of environmental or other genetic influences, and that this number found by Penrose and his co-workers (1948), by Woolf (1955), and by the author, no matter what their controls, may repre-

sent a close approximation to the truth in the populations they studied

Finally, if Dr. Schull objects to the formal studies on human cancer since they do not deal with a "defined population," one is tempted to ask if the patients observed by an individual clinician are a better "defined" population epidemiologically, so that their impressions should be accepted in preference to the "chunks of data" collected by various scientific workers.

ACKNOWLEDGMENTS

This work has been sponsored by the American Cancer Society in the beginning and by the United States Department of Health, Education, and Welfare, Field Demonstrations and Investigations Branch. To both of these organizations, I give my thanks

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Genetic Studies of Families with High Cancer Incidence

CLARENCE P. OLIVER, PH.D.

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A number of rare human cancers are genetically determined. Retinoblastoma is generally considered to have a dominant inheritance. Sometimes, however, a person who apparently has the gene does not develop the cancer. Intestinal polyposis is a precancerous condition with an hereditary basis. A child with xeroderma pigmentosum develops skin cancer after exposure to light, one pair of genes is responsible for the highly sensitive skin.

Cancers of the stomach, colon, uterus, and breast cannot be considered uncommon. The genetics of these disorders is difficult to unravel. Excellent reviews of studies in this field may be found in the reports of Woolf (1955) and by Anderson, Goodman, and Reed (1958). Reports to which I refer are to be considered only as examples, and are made without purposeful intent to deny any investigator his just due.

STATISTICAL ANALYSES

One method of studying genetically determined cancer has been to make statistical analyses of cancer occurrence among relatives. The frequencies with which relatives have breast cancer are compared with expected frequencies on the assumption that the patients' relatives face the population risk. Expected frequencies may be calculated by the use of proportional mortality or morbidity rates.

Penrose, Mackenzie, and Karn (1948) used probands from among

women patients in London hospitals. Woolf (1955) obtained probands by going to death certificates for persons who had died in Utah during a ten-year period. Anderson, Goodman, and Reed (1958) used patients of the University of Minnesota tumor clinic. The three groups of probands represent population units from widely separated geographic areas. Differences in the observed frequencies of breast cancer in mothers and sisters of one proband group in comparison with the other two sets are not so important for purposes of the studies Comparisons were made of the frequencies in relatives with the population risk for that specific area. The three investigators are in agreement that breast cancer in close relatives of cancer probands occurs more frequently than would be expected if the families faced the population risk.

Comparisons have also been made between the frequencies of breast cancer in relatives of cancer patients and in relatives of control probands Investigators have used great care in choice of cancer and control probands. Jacobsen (1946) used cancer cases selected from the records of the Danish Cancer Registry. Probands used by Woolf (1955) and Anderson, Goodman, and Reed (1958) have been mentioned. Macklin (1954) used patients of the Cancer Clinic and the University Hospital at Ohio State University. Jackson and Oliver (Oliver, 1958) used private patients with breast cancer who came to Dr. Jackson for examination and were found to have cancer.

Choice of control probands becomes a difficult task. Actually we do not yet know what is a good control. One investigator will seek controls from among patients who have gone to a physician for an illness other than cancer or tumor Another will prefer controls from among persons who are not ill. Every worker can pick flaws in the choice of controls by other investigators. In fact, he may not be too pleased with his own choice, after he takes a second look. Once a choice has been made the investigator faces other disturbing conditions. Probably the persons in the chosen category will be fewer than in the cancer probands. Further, it is not always possible to get the willing co-operation of the control group's relatives, although most relatives of cancer probands will help in the investigation.

The completed investigations show that close female relatives of cancer probands have a higher frequency of breast cancer than do the corresponding relatives of control probands Breast cancer shows a concentration in some families. The explanation may be that heredity is a causal factor, although gene action alone does not seem to be responsible for the phenomenon.

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generations are not available. Family members may know that a relative had cancer, particularly if the breast was involved. Consequently, the investigator who questions enough of the family members may feel justified in concluding that a particular person had breast cancer even though the pathological record is not available.

Information about relatives among the group belonging to the patient's and to her children's generations will have been collected and kept under conditions better than those for past generations. That statement is, of course, to be altered when some geographic areas are used and some families are studied. The living generations, together with the reliable data about the past, and follow-ups in the future can give us an understanding of the pattern of inheritance and the environmental agents responsible for cancer development. However, with the present therapy and the probable advance in the future, a person with the potentiality to develop breast cancer may never do so. A precancerous breast condition may cause the surgeon to remove the tissue, or otherwise to prevent the change to obvious malignancy. We all hope that this does happen. For purposes of genetic study we can also wish that the records will show the precancer and not just record the lack of cancer.

Multiple Cancers in a Family

Any family history with a large number of cancerous members will attract attention. It is more likely to appear in the literature than will a history with few or no affected members. Pedigrees can be more useful in genetic studies if they include families with few cancer cases as well as those with many cases. Pedigrees showing many cancerous relatives with all sites involved will be of little value in genetic studies, although they may be of interest. Histories with a concentration of cancer of a specific site present patterns that make it possible for an investigator to determine the genetics of the tendency. Actually, though, the genetic interpretation cannot be made if only a few family histories are available, and families with a high concentration of cancer at any single site are not common.

Hauser and Weller (1936) reported on a family which Warthin had studied at an earlier date. This record was the first to call attention to familial concentrations of cancer of particular sites. The family had 174 members who had reached age 25. Among these members, 14 9 per cent had developed cancer of the gastrointestinal tract, and 8.6 per cent had had cancer of the uterus Only two other kinds of cancer were reported for the group; one involved the breast;

Twin Studies

Twin studies have been used to measure the relative effects of heredity and environment as agents responsible for cancer occurrence. Monozygous twins are genetically identical. Consequently, a pair should be alike for cancer if gene action is responsible for the potentiality. Common environment may also cause the pair to develop cancer. Dizygous twins may have the same pair of genes, but the probability is no greater than that for regular siblings.

Gorer (1958) analyzed the twin data reported by Busk, Clemmesen, and Nielsen and by von Verschuer and Kober. Not all pairs of monozygous twins showed concordance. They did show a higher degree of concordance for similar site involvement than did the dizygous twins. Macklin (1940) reported at an earlier date that monozygotes and dizygotes do not differ in concordance to any considerable extent *when all cancers are treated as a single entity*, but that concordance in similar site involvement is markedly higher for the monozygotes. Monozygous twins may show discordance at the time they are reported, but the unaffected member may develop cancer at a later time.

Very few reports have been made of pairs of twins with both having breast cancer. If the gene is uncommon, as Gorer (1958) suggested, monozygous twins with both members affected will be a rare occurrence. Follow-up studies should be made of any pair of twins of whom only one has cancer, whether the pair be monozygotes or dizygotes.

Family Histories

Pedigrees of families with cancerous members should be used more frequently than has been done in the past. The survey type of study indicates a probable genetic basis for breast cancer potentiality. Pedigrees will be the logical method to use to determine the pattern of distribution within a family and the reason for the observed distribution.

The investigator should get a complete and accurate record of all family members. Obviously a point will be reached where the return does not justify the effort. Information about relatives, direct or collateral, who lived in the grandparent and great-grandparent generations cannot always be accurate. Not only is the diagnostic skill of today better than it was in the past, but records often have been lost, if they were kept at all. In some geographic areas, records of past

generations are not available Family members may know that a relative had cancer, particularly if the breast was involved. Consequently, the investigator who questions enough of the family members may feel justified in concluding that a particular person had breast cancer even though the pathological record is not available.

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the other, the face. Gardner and Stephens (1950) and Woolf and Gardner (1951) have reported the distribution of breast cancer in families. Gardner and Stephens reported that nine breast cancer and four benign breast cases occurred in three of eight possible branches of a family group. Gardner (1954) checked the family at a later time and discovered that one new breast cancer and two benign breast cases occurred in members of those same branches. Woolf and Gardner in their study of another family found that eight breast cancer cases were descendants of one person. The siblings of this individual had only a few descendants with cancer.

Frequency of Multiple Cancer Histories

Family histories with multiple cases of breast cancer will not be found very often. At the time she is interviewed, a proband's close relatives are more likely to be free of, than to have had, breast cancer. If the distant relatives are included, more probands will show positive histories.

In the records collected by Dr. Jackson and myself, we found a striking number of probands whose sisters, mothers, and aunts have not had breast cancer. Among 316 probands, five were not used in the studies of familial tendency. Four of the five are sisters of four other probands. The other was adopted and we have no history of her family.

The numbers of probands whose sisters, mothers, and aunts were free of or had breast cancer are shown in Table 1. The relatives have

TABLE 1. Breast Cancer in Sisters, Mothers, and Aunts of Probands

History	No probands	No relatives age 30 or over			
		S	M	MA	PA
No B ca	247	511	246	414	340
B ca	64	147	64	155	152
Total					
relatives		658	310	569	492
B ca	311	25	19	19	16

One mother d age 25.

S, sister; *M*, mother; *MA*, maternal aunt, *PA*, paternal aunt, *B*, breast, *ca*, cancer.

been used in this report only if they lived to at least age 30 years. One mother died at age 25. Among the 311 breast cancer probands, 247 had no history of breast cancer in her close relatives, that is, the sisters, mothers, and aunts, although some of the probands had close relatives with cancer at other sites. Thirty-seven of the 247 probands had grandmothers, cousins, or other distant relatives with breast cancer. Twenty-five of 658 sisters, 19 of 310 mothers, 19 of 569 maternal aunts, and 16 of 492 paternal aunts have had cancer of the breast All of these affected relatives were members of 64 family groups, representing 20.6 per cent of the probands.

Among the 64 probands with affected close relatives, age 30 years or older, the majority have so far had only one relative who developed breast cancer. Fifty-two had only one breast cancer relative; ten had two; one had three; and one had four affected relatives. The distribution and numbers of affected members by degree of relation-

TABLE 2 Combinations of Breast Cancer in Sisters, Mothers, and Aunts Age 30 Years or Older

Combination	No probands	Sisters		Mothers		M aunts		P. aunts	
		No	ca	No	ca	No	ca	No	ca
S+M	3	9	5	3	3	4	0	2	0
S+MA	2	8	2	2	0	8	3	3	0
M+MA	2	0	0	2	2	5	2	3	0
MA+PA	1	3	0	1	0	3	1	3	1
S only	2	5	2	2	0	0	0	0	0
	12	39	14	12	0	26	0	28	0
	1	6	2	1	0	2	0	0	0
M only	1	1	0	1	1	0	0	1	0
	3	5	0	3	3	10	0	0	0
	3	0	0	3	3	5	0	11	0
	7	20	0	7	7	18	0	23	0
MA only	11	25	0	11	0	39	12	31	0
	1	4	0	1	0	2	1	0	0
PA only	2	0	0	2	0	0	0	4	2
	13	22	0	13	0	33	0	43	13
Totals	64	147	25	64	19	155	19	152	16

S, sister, *M*, mother, *MA*, maternal aunt, *PA*, paternal aunt, *B* breast; *ca*, cancer.

ship are shown in Table 2, which also identifies the number of probands lacking sisters or aunts.

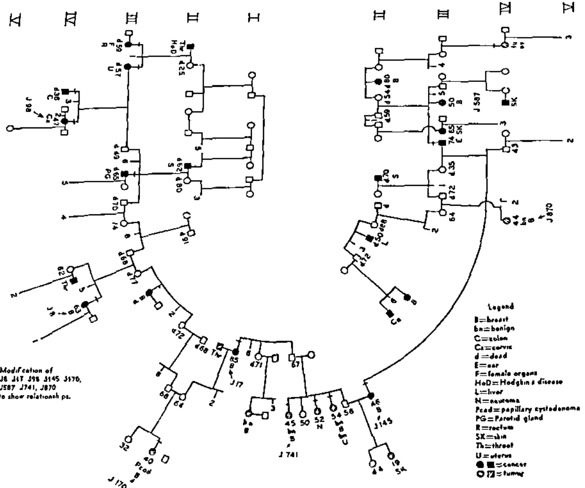
The proband with four affected close relatives had three of five sisters and a mother with breast cancer; one paternal and two maternal aunts were free of the disease. One proband with three breast cancer relatives had one of five sisters and two of six maternal aunts with the disease; two other sisters had cancer of the cervix or colon, and one of two paternal aunts had cancer of the uterus. The ten cases having two affected members each had the following affected pairs: two with a sister and mother, two with a mother and maternal aunt, one with a maternal and a paternal aunt, one with a sister and maternal aunt, one with two maternal aunts, and three with two sisters. The number of the close relatives with breast cancer for each degree of relationship is given in the table. The number of affected sisters differs with that given by Oliver (1958) because of the age difference used in the two reports.

There is one way to increase the number of family histories with multiple cases of breast cancer in the kinships. This method was used in our study. Rather than making just a survey of the affected relatives at the time patients were interviewed, Jackson and I have added new cases when "normal" relatives were discovered to be cancerous or developed cancer after the original survey. At the same time, members reported by the proband or relatives to have breast cancer were eliminated from that category if evidence established that they were not affected. The addition of data has been made possible because Dr. Dudley Jackson, Sr. was an active collaborator. He obtained the co-operation of the patients and their families and made follow-up studies of the patients. Without this co-operation the study would have been only a survey.

By our method we also have been able to combine families of two or more probands when relationship has been established. Family files identify members by surname and given name. A new cancer case, whether it is a proband or another person, can be identified as to relationship by consanguinity or marriage to other probands.

Figure 1 is an illustration of how families can be linked by proper records. It shows the relationships among eight probands who had breast cancer, cancer of another site, or a benign breast disorder The number of relatives of the eight probands total 381 and cannot all be

Figure 1 Relationships among eight probands and their affected relatives


Legend
B=breast
bn=benign
C=colon
Cx=cervix
d=dead
E=ear
F=female organs
HoD=Hodgkin's disease
L=liver
N=neuroma
Pcad=papillary cystadenoma
PG=Parotid gland
R=rectum
SK=skin
Th=throat
U=uterus
=cancer
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Modification of
J8 J17 J98 J145 J170,
J587 J741, J870
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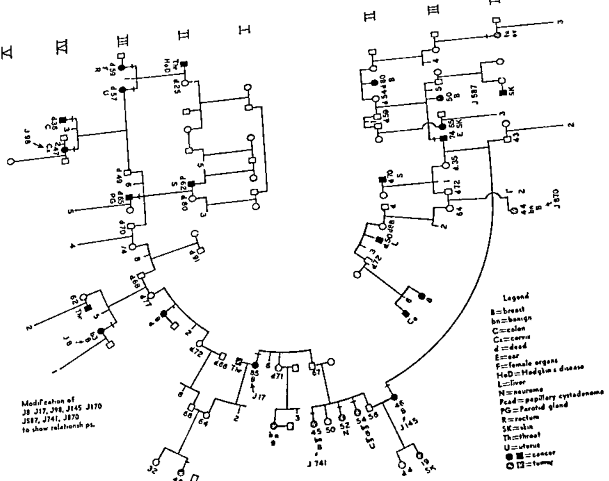
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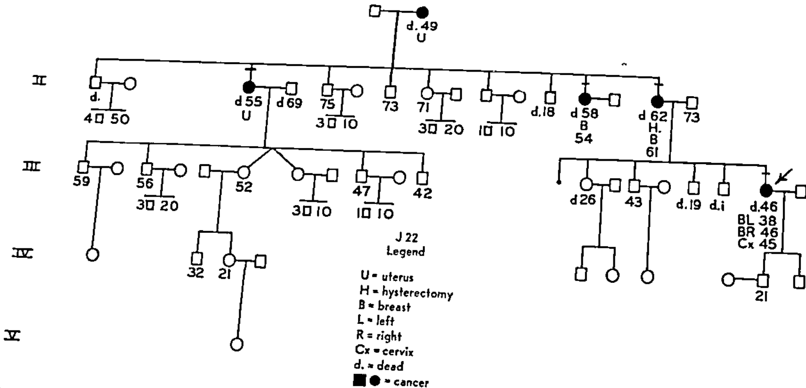


Figure 2 History of cancer among relatives of breast cancer proband having multiple primary site involvements.

shown in the figure. Some of them have not been verified as to normalcy or, in a few cases, cancer.

Every proband shown in Figure 1 is not related to all other probands. J 98 is not a breast cancer proband. Instead, she had cancer of the cervix. She had had no relative with breast cancer, although six (four proved cases) have had cancer at other sites. She is not related to the other seven probands except by the marriage of an uncle to a relative of J 8. Although J 98 does not need to think, genetically, about cancer in J 8 and her relatives, a descendant of the uncle of J 98 would need to consider relatives of J 98 and also J 8.

J 8 had breast cancer. One of her maternal aunts had breast cancer and another has a granddaughter, case J 170, with a benign breast condition. J 170 is related genetically to J 8 and her affected relative. Her maternal grandfather was reported to have had cancer of the throat (unproved) and her maternal grandmother (J 17) has had breast cancer. Three relatives, through J 17, show benign breast histories, including J 741, and one had a neuroma. This family is related by marriage to J 145.

J 145 has had breast cancer. Her relatives include two with breast cancer histories, one with benign breast involvement (J 870), and three with cancer or benign tumors at other sites. The daughter of J 145 must consider her mother's relatives and also J 17, J 170, and the affected bloodline relatives of J 17.

Patients with bilateral breast cancer apparently are no more likely to have close relatives with breast cancer than are patients with unilateral cancer. Twenty of the 311 probands used in our studies of positive family histories had bilateral breast cancers. Four of the 20 had sisters, mothers, or aunts with the disease This frequency is practically identical to the frequency of unilateral cases with affected close relatives.

A history of one patient with multiple site involvement who also had close relatives with breast cancer is given in Figure 2. The proband had cancer of the left breast at age 38 and cancer of the right breast at age 41. She then had cancer of the cervix at age 45 and died one year later. Among the patient's relatives, the mother had unilateral breast cancer at age 61 and died one year later. She had had a hysterectomy at an earlier age A maternal aunt with unilateral breast cancer at age 54 died at 58. One of the other two maternal aunts died with cancer of the uterus at age 55. The maternal grandmother was reported to have died at age 49 with cancer of the uterus.

Pedigree studies can give us information about the distribution pattern of breast cancer in family groups. The concentration in branches of families may make possible an interpretation of the method of inheritance as well as the environmental agent involved in cancer occurrence. However, the number of families with relatives of probands also having breast cancer will be small. Only 20.6 per cent of probands in our studies had one or more close relatives with breast cancer. Only 32.4 per cent had any relative reported to have had breast cancer.

Multiple cases of cancer among relatives may be discovered even though a proband's history fails to show them. This can be accomplished by filing names and cross matching names of breast cancer patients. One example collected in this manner shows eight probands with cancer or benign tumor who are related by consanguinity or by marriage Although one person in the composite group may have few relatives with cancer, other persons have to consider the relatives of two or more probands. Bilateral breast cancer patients do not have any higher frequency of cases among relatives than do unilateral cases. Only four of 20 bilateral cases had close relatives with breast cancer One example is given.

The high frequency of solitary cases may be due to the existence of two groups of causes for breast cancer, some being hereditary and others environmental We should also consider the possible interrelationship between genes and an unknown environmental agent in which the gene may be present but unexpressed unless the proper environment coexists. Such temperamental genes are known in experimental organisms, and instances can be cited in man.

Pedigree and twin studies offer us methods for determining the weight of heredity in breast cancer If heredity is a factor, as it seems to be, studies must be made to determine whether recessive genes or dominant genes with reduced penetrance are concerned. Follow-ups of available histories will be the logical procedure to follow.

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Only the latter case has not been medically verified. Her doctor had died and the records were not available.

What is responsible for the high proportion of probands with no close relative having breast cancer? The problem may seem baffling, particularly when we believe that breast cancer tendency has a genic basis. There is *always the possibility that family records* have not been complete and that all breast cancer cases have not been reported. In our studies, however, an attempt was made to get complete information about all relatives, those reported to have been free of cancer as well as those reported to have had cancer.

A genetic trait cannot be expected to occur always in a Mendelian ratio. Most likely the hereditary basis, if inheritance is involved, is not that of a simple dominant because too often mothers of probands are free of cancer. A summary of the data reported by a number of investigators on breast cancer frequency in mothers of probands is given by Anderson, Goodman, and Reed (1958, Table 41). The reported frequencies range from 11.6 to 2.5 per cent. In our own studies (Oliver, 1958) the frequency was found to be 6.1 per cent. Fathers of some probands could have transmitted a dominant gene for the tendency. However, the frequency of affected mothers would still be lower than might be expected if a dominant gene were involved. A recessive gene, not too uncommon in the population, should be considered. The possibility that a gene could have come from the father is shown by the relative frequencies of breast cancer in maternal and paternal aunts. In our records of aunts who lived to be 30 years or older, 19 of 569 maternal aunts and 16 of 492 paternal aunts of the 311 probands used in the studies were reported to have had breast cancer. The frequencies are the same, approximately 3.3 per cent. Aunts were discarded from the record if the information seemed unreliable. The frequency in sisters, age 30 or older, of 311 probands was 3 8 per cent. The frequencies in the 64 multiple case kinships are, of course, higher. The possibility that a recessive or a dominant gene with reduced penetrance is involved merits further study. More completed family histories must be available before the pattern can be determined.

Conclusion

Statistical studies of breast cancer frequency in relatives of cancer probands compared with expected frequencies indicate a genetic basis for breast cancer. Twin studies also show that heredity is a factor for site involvement. The disease has no simple genetic basis.

Symposium Summary

HOWARD B. ANDERVONT, SC.D.

National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health, Education and Welfare, Bethesda, Maryland

These summarizing remarks are presented in memoriam to Dr. Arthur Kirschbaum, a late member of this institution. I'm sure that all of us who had the privilege of knowing him and appreciated his work were thinking of him while preparing for, and while attending, this Symposium.

Those familiar with the major advances in cancer research will look upon this Symposium as a tribute to the contributions of geneticists. While these achievements are fundamental to all fields of biology, they are so important to medical science that they can be used as a framework for this summation.

The first and perhaps the most important contribution was the development of inbred strains of experimental animals for cancer research Before an audience of geneticists and cancer workers it is not essential to emphasize the significance of this contribution, but I should like to point out that the development within a species of inbred strains showing pronounced variations in susceptibility to naturally occurring diseases, such as different types of cancer, was indeed a major accomplishment.

The importance of inbred animals has been accepted by cancer investigators, it is difficult to conceive a basic approach in which they are not essential We have heard several participants who carried out genetic analyses, and all used inbred animals because they are indispensable for such work. One participant was concerned with the site

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of gene action in the occurrence of tumors and showed that, regardless of the carcinogenic stimulus, the response of the host is largely dependent upon genic control of tissues which become malignant; even in tumors resulting from the interplay of different physiological stimuli, each stimulus is under genic control. Another used inbred strains to study the immunogenetics of a sex influence in the viability of intrastrain skin grafts. While male-to-female incompatibility probably is primarily an immunological problem, its solution demands the use of inbred animals. Another speaker discussed the acquisition of drug-resistant lines of transplantable tumors. Nearly all experimental animals used in these studies were, of necessity, from inbred strains, and the consensus is that mutation or selection and adaptation of tumor cells play major roles in the reaction.

One investigator exposed cells in tissue culture to different culture media and found that hereditary metabolic variants could be isolated from the parental strain. The strain of cells used was from an inbred mouse. Other work with tissue cultures from the same inbred strain has shown that, starting with a clone of tumor cells derived from a single cell, lines of cells were obtained which showed wide variations in their transplantability back to inbred mice, in their metabolic activities, and in their chromosomal aberrations. This work with tissue culture emphasizes the desirability of using cells from inbred animals when studying *in vitro* changes to malignancy, because proof of the presence of tumor cells rests upon the ability of the cells to produce tumors when implanted into suitable hosts.

The variations of cell strains procured by tissue culture techniques are similar to changes observed in transplantable tumors when they are transferred through serial passages. Single passages through F_1 hybrids have been shown to alter permanently the growth characteristics of tumors. This presents the opportunity to mention early in this summation one important basic feature of cancer. This feature is the remarkable individuality of tumors, including those derived from the same tissue. Furthermore, as expressed by several speakers, transplanted tumors and spontaneous tumors undergo unpredictable and random changes. This implies that the development and growth of cancer are a series of complex interactions between the host, the carcinogenic stimulus, and the environment, and that the ultimate solution of the problem will not rest upon the contributions of any one scientific discipline. For example, inbred mouse strains vary in their susceptibility to spontaneous liver tumors; and in the strains thus far studied, males are more susceptible than females to the de-

velopment of these tumors. When members of one high tumor strain receive a tumor-inducing azo dye, the females are far more susceptible to tumor induction than the males, but the sex difference in response can be altered by castration or exposure to sex hormones. Both sexes, exposed to another tumor-inducing chemical, respond by developing liver tumors. Diet also exerts a definite influence upon the occurrence of tumors in the same mouse strain. Thus, the interplay of chemical compounds, hormones, and dietary factors elicits a variety of responses in animals with identical genetic constitutions.

The second basic contribution to cancer research made by geneticists was the demonstration that genetic factors controlled the growth of transplanted tissues. This discovery laid the foundation for all subsequent research with transplantable tumors and had a wider application in the practical problem of normal tissue transplantation.

Over 30 years ago geneticists formulated the rules for the successful growth of transplanted tumors. They also found that such tumors could undergo sudden changes in growth characteristics and showed, by application of the F_2 progeny test, that the ability of a tumor to grow progressively in a wider range of hosts was accompanied by a decrease in the number of its genetic factors. This earlier work is pertinent to this summation because, if my memory is correct, these changes in growth properties were attributed to mutations occurring in the tumor cells. To those investigators who were not geneticists, such indication of mutation in cancer cells was considered evidence that normal cells could also mutate. It was understood by oncologists that direct genetic analysis of malignant cells was not possible, but acceptance of the mutation theory of cancer depended to a great extent upon whether geneticists agreed that somatic mutations could occur.

We have heard frequently, and at this meeting, that the two present-day leading theories of cancer genesis are the virus theory and the mutation theory. Biologists interested in the cancer problem have long since accepted the fact that viruses can cause cancer. The problem has been how viruses induce malignancy, and the argument has been whether they are responsible for all cancers. The same workers also have accepted various definitions of cancer cells as "permanently altered cells" or "transformed cells." No individual can speak for all oncologists, but it is believed that the majority of those who are not geneticists will accept the proposition that cancer cells arise through the process of a single or a series of somatic mutations. Indeed, it is problematical whether general acceptance of the somatic mutation

of gene action in the occurrence of tumors and showed that, regardless of the carcinogenic stimulus, the response of the host is largely dependent upon genic control of tissues which become malignant; even in tumors resulting from the interplay of different physiological stimuli, each stimulus is under genic control. Another used inbred strains to study the immunogenetics of a sex influence in the viability of intrastrain skin grafts. While male-to-female incompatibility probably is primarily an immunological problem, its solution demands the use of inbred animals. Another speaker discussed the acquisition of drug-resistant lines of transplantable tumors. Nearly all experimental animals used in these studies were, of necessity, from inbred strains, and the consensus is that mutation or selection and adaptation of tumor cells play major roles in the reaction.

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Further exploration of the cytogenetics of tumors will do much to reveal whether chromosomal aberrations are intricately bound to the cause of cancer or whether they are a manifestation of the cancer process. Such studies are fundamental because they are concerned with the activities of normal and neoplastic cells. Perhaps more study of the chromosomal patterns of the same type of primary tumor induced by a variety of carcinogenic agents in experimental animals of the same genetic makeup may be fruitful.

The third major contribution by geneticists which was fundamental to cancer research was the discovery of the mouse mammary tumor virus. Cancer of the mammary gland in mice has been studied intensively for many years and, prior to the discovery of the virus, the importance of heredity and hormonal stimulation in its occurrence was firmly established. Discovery of the virus added a third influence to the factors of heredity and hormones. All three are important, and it is the consensus that a tumor can arise when a deficiency in one influence is compensated by an increase in one or both of the others. This is pertinent to the tumor virus problem because this virus, instead of being the sole object of attention, is looked upon as part of a complex interplay of forces leading to malignancy.

The only known lesion indicative of the presence of the virus before gross tumors appear is the occurrence of hyperplastic nodules in the mammary glands. But hyperplastic nodules are also found in the glands of old mice that are presumably free of the virus. This implies that the role of the virus may be to provide these nodules in which the tumor develops and, if this is true, then the function of the virus in tumor production may be that of an accelerator. Briefly, it may act indirectly by producing, at an early age, a benign lesion, which under suitable hereditary and hormonal conditions becomes neoplastic. This, in turn, implies that any virus capable of producing benign and lasting hyperplasia could be implicated in the origin of cancer. A very interesting paper presented at this meeting dealt with this problem. By means of an ingenious technique, the nodules or normal glands were transplanted to a site where their further development took place. The nodules produced tumors, and this established their preneoplastic nature. Other efforts to establish this preneoplastic nature had met with little or no success. It also was shown that the nodules and normal mammary tissues differ in their sensitivity to hormonal stimulation, thereby suggesting an indirect action of the virus. Further, some nodules produce a variety of outgrowths which appear to be stable upon serial transplantation. This presents

theory would alter the course of cancer research, because the problem would then be to learn the cause of the mutation and how it is brought about.

We heard one excellent paper in which this problem was presented. Apparently a number of known processes can bring about genetic variation in somatic tissues, and the speaker suggested more study of nuclear differentiation which precedes the somatic change.

Geneticists have made remarkable progress in the study of tumor transplantation. They found that one genetic locus is of considerable importance in determining susceptibility to tumor transplants, and isogenic-resistant lines of animals have been developed which are invaluable for studies of tumor biology. One of our speakers who used *isogenic lines to study the cytogenetics of transplanted tumors found* that each tumor was individualistic and each showed characteristic remodeling of chromosomes. While this remodeling may be involved in the development of malignancy, it is not necessarily the predominant cellular change, since chromosomal changes may not occur in virus-induced tumors. Another investigator studied chromosomal alterations in cultures of embryonic tissues to ascertain whether the alterations were related to the change to malignancy. Deviations were seen at the first explantation, but ultimately, as with transplanted tumors, stem lines emerged. One developed in the cells *in vitro* and one in cells that produced tumors when reimplanted into mice. Thus, malignancy produced in tissue cultures may be due to mutations occurring during the process of adaptation. Attention was again directed to the possible absence of chromosomal aberrations in virus-induced tumors, and reference was made to a recent publication in which investigators report finding a conspicuous heteropyknotic chromosome in cells from 18 of 19 primary breast tumors of mice. These tumors arose in mice carrying the mammary tumor virus. This observation may be of considerable importance if it is confirmed in other virus-induced tumors, provided that the chromosomal alteration is not found in breast tumors of mice that are free of the virus.

Another worker studied chromosomal status in drug-resistant sublines of a transplantable tumor. A large submetacentric chromosome was present in the parent line and in four sublines, all of which were *sensitive to a drug*; whereas five resistant sublines did not carry the chromosome. This suggests that genetic changes may underlie the metabolic changes which accompany the acquisition of resistance to this particular drug. No other consistent cytological correlation was observed among the nine sublines with respect to five other drugs.

virus associated with it. The two findings together suggest not only that the virus is intimately associated with the cancer cell but also that the association is concerned with the species character of this cell, since these immunologic reactions are ordinarily species specific. Immunologically, the mouse mammary cancer cell appears to be a mouse cell with a substituted virus species."[1]

The important thing here is that the virus theory and the mutation theory of cancer genesis are converging This convergence has intensified studies of nucleic acids because these compounds are now considered essential components of viruses and chromosomes. On this program, one paper described an association between the presence of the mammary tumor virus and RNA. One investigator studied the relationship between DNA metabolism and carcinogenesis and reported that the results supported cytological findings of differences between normal and malignant tissues. Others studied the sequence of events in ultraviolet-induced mutation in bacteria and concluded that their results suggested involvement of RNA synthesis in genetic replication, in that the mutagenic changes occur primarly in RNA and then are transferred to the genic material during DNA synthesis.

One speaker presented a scholarly dissertation of the pathways by which radiation damage could result in cancer or mutations. Direct, induced mutations, and indirect changes involving release of a provirus or a reaction akin to transduction, could result in cancer.

The remaining papers can be divided into two groups The first consisted of discussions of experimental work which is fundamental to the entire field of genetics One speaker investigated the genetic area controlling the formation of a specific enzyme and suggested that the genetic area can be resolved into a number of discrete functional units. Also, a genetic change can produce not only an altered enzyme, but also quantitative changes in enzyme formation. Another paper in this group consisted of a discussion of an hypothesis concerning the possibility that cytoplasmic particles, related to or closely resembling virus or provirus, can mutate into a virus or provirus and give rise to cancer.

The second group of papers dealt with the practical problems of heredity and cancer. One author studied bovine ocular carcinoma and found a definite genetic basis for susceptibility to this disease. The final four papers were concerned with human genetics or genetic studies of human cancer. One investigator reviewed the accomplish-

[1] Green, Robert G. 1947. The Species Character of Cancer Cells *Science*, 105 93

an opportunity to learn whether nodules containing virus differ from those arising in virus-free mice.

You are aware of the recent interest in cancer viruses evoked by studies of mouse leukemia. Various workers have reported the detection of viruses in leukemic and other tissues, but one of the most interesting viruses exposed during this work is one that, thus far, does not produce leukemia but induces a distinctive tumor of the parotid glands as well as a wide variety of other tumors and lesions. It possesses many attributes of other non-cancer-inducing viruses. It can be cultured in tissue cultures, it elicits antibodies which permit its detection, and, when introduced into a colony, it is spread from mouse to mouse by way of infected saliva and urine. Further work on the properties and epidemiology of these newly discovered viruses will influence cancer research in the years to come because the interest of virologists in the cancer problem has been aroused. This is pertinent to the purpose of this Symposium, because if cancer cells are derived from normal cells through the process of somatic mutation, then the viruses must be involved in the reaction.

This program included a brilliant exposition of the biological properties of temperate bacteriophages, of their relationship to the cells they infect, and of how their characteristics are applicable to a genetic concept of the cancer process. A virus could infect a cell as a prophage infects a bacterium—gain access to the chromosomes and remain integrated with the cell genome—or it might, through the process of transduction, transfer genetic characters from cell to cell. Virologists interested in cancer have long postulated the existence of "masked," "latent," "quiescent," or "toothless" viruses and an "enduring relationship" between viruses and cells. Recently I read again the following paragraph written over ten years ago by one such investigator.

"There are two indications in our data that point to some extremely close relationship, or combination, of the virus and the cancer cell. The first is that in absorption tests the virus antibodies combine with the cancer cell but do not combine with the normal cell of the same kind. While this finding demonstrates a specific absorption of immune bodies by the cancer cell, the absorption could be due to free virus contained within it. The second is that the virus immune bodies have an *inactivating, or lethal, effect upon the cancer cell,* which would seem to mean that some virus is not free but occupies a vital position in the cancer cell. The malignant animal cell seems to have become completely dependent upon the vital activity of the

pants and the audience, our thanks to the staff of The University of Texas M. D. Anderson Hospital and Tumor Institute for this meeting. These Symposia on Fundamental Cancer Research have become a major annual event. Congratulations are extended to the Chairman, Dr. Felix L Haas, and to his Symposium Committee, who were responsible for the program. They have carried us from the origin of genic material through the gene, chromosome, cytoplasm, nucleus, cell, tissue, and organ to the entire organism. The only omission of any consequence was a discussion of genetic factors in the hormonal aspects of cancer research, but I noticed this only because it restrained me from extolling the contributions of geneticists in this branch of research. Certainly the objective of showing that the discipline of genetics bears directly upon all fundamental cancer research has been achieved in this Symposium.

ments in human genetics during the last 50 years and described how accumulated knowledge of genic control of metabolic processes has made medical genetics an important part of modern medical science. Another informed us of the problems in the study of human genetics and in the establishment of procedures for the evaluation of genetic and environmental factors.

Two papers described efforts and the results obtained in an approach to the difficult problem of the influence of heredity in human cancer. Those who have engaged in cancer research and benefited through the use of inbred animals will attest to the importance of genetic factors in the occurrence and growth of cancer. This, together with the fact that most diseases, in common with many biological phenomena, reflect the response of an organism to its environment, make it highly improbable that cancer could be the great exception. The question is not whether the host is involved in the cancer process, but why and how the host is concerned. From the earliest days of cancer research, those who probed this problem have recognized the importance of the host's constitution.

Two findings have emerged from the experimental approach that may explain in part at least the difficulties encountered when evaluating genetic influences in a mixed population. The first finding is that genetic factors control the *degree* of susceptibility to cancer development: Susceptibility is not an all-or-none proposition. Inbred animals exhibit wide variation in their responses to a carcinogenic stimulus, but with suitable procedures even the most resistant develop tumors. When animals from a highly susceptible strain are exposed to the same stimulus, all members do not develop tumors at precisely the same time. The second finding is that within an inbred strain, susceptibility is limited to certain kinds of cancer; thus far an inbred strain susceptible or resistant to all cancers has not been established. One strain of mice is susceptible to the breast tumor virus as well as to spontaneous and induced liver tumors but is resistant to chemically induced skin cancer; whereas another is resistant to the first two tumors but is susceptible to the third. Likewise, a strain may be resistant to breast cancer induced by the virus but susceptible to the same tumor induced by a chemical. When differences in hormonal stimulation, dietary habits, and environment are superimposed upon genetic susceptibility, it becomes obvious that, within a heterogeneous population, genetic factors would have to exert a decided influence before they could be detected easily.

In conclusion, it is a pleasure to express, on behalf of the partici-

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